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(54) Title: ANTIBODY FORMULATION

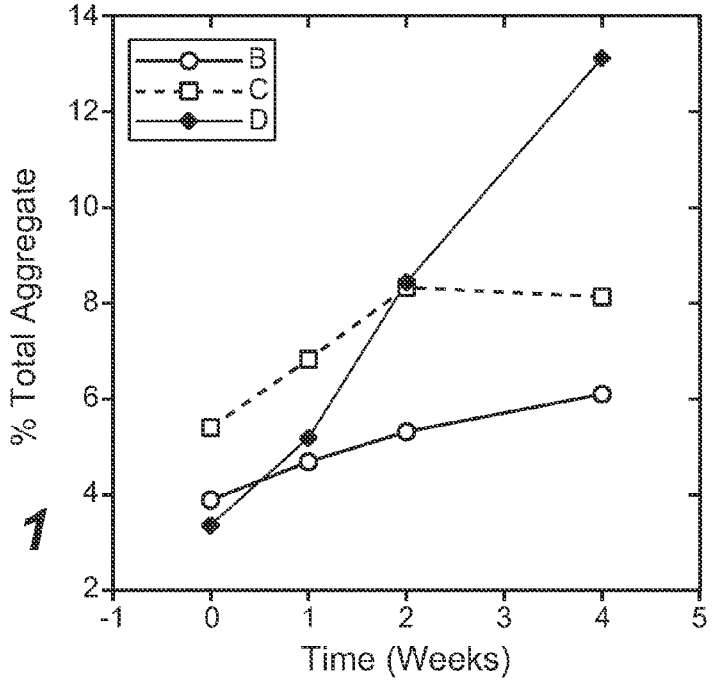

FIG. 1

(57) Abstract: The invention provides a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody, optionally, not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.0 to about 6.0, and an optional surfactant, methods for making such a formulation, and methods of using such a formulation.

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Antibody Formulation

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/288,535, filed December 21, 2009, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] This invention is directed to a stable aqueous pharmaceutical formulation comprising an antibody.

BACKGROUND

[0003] In the past years, advances in biotechnology have made it possible to produce a variety of proteins for pharmaceutical applications using recombinant DNA techniques. Because proteins are larger and more complex than traditional organic and inorganic drugs (e.g., possessing multiple functional groups in addition to complex three-dimensional structures), the formulation of such proteins poses special problems. For a protein to remain biologically active, a formulation must preserve intact the conformational integrity of at least a core sequence of the protein's amino acids while at the same time protecting the protein's multiple functional groups from degradation. Degradation pathways for proteins can involve chemical instability (e.g., any process which involves modification of the protein by bond formation or cleavage resulting in a new chemical entity) or physical instability (e.g., changes in the higher order structure of the protein). Chemical instability can result from deamidation, racemization, hydrolysis, oxidation, beta elimination or disulfide exchange. Physical instability can result from denaturation, aggregation, precipitation or adsorption, for example. The three most common protein degradation pathways are protein aggregation, deamidation and oxidation. Cleland et al *Critical Reviews in Therapeutic Drug Carrier Systems* 10(4): 307-377 (1993).

[0004] Included in the proteins used for pharmaceutical applications are antibodies. An example of an antibody useful for therapy is an antibody which binds to anti-VEGF. There is a need in the art for a stable aqueous pharmaceutical formulation comprising an antibody, such as an anti-VEGF antibody, which is suitable for therapeutic use.

SUMMARY

[0005] The invention provides stable aqueous pharmaceutical formulations comprising a therapeutically effective amount of an antibody, optionally, not subjected to prior lyophilization, a buffer maintaining the pH in the range from about 4.0 to about 6.0, and an optional surfactant, methods of making the formulation and methods of using the formulation.

[0006] One embodiment of the invention provides a stable aqueous pharmaceutical formulation, the formulation comprising a therapeutically effective amount of an antibody in an arginine buffer, pH 4.0 to 6.0. In some embodiments, the buffer is an arginine acetate buffer, pH 4.5 to 5.5. In some embodiments, the buffer is an arginine acetate buffer, pH 4.8 to 5.4. In some embodiments, the buffer is an arginine acetate buffer, pH 5.2. In some embodiments, the arginine actetate concentration in the buffer is from about 25 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 50 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 75 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 100 mM to about 250 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 120 mM to about 240 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 150 mM to about 225 mM. In some embodiments, the arginine acetate concentration in the buffer is about 200 mM. In some embodiments, the formulation further comprises a surfactant. In some embodiments, the surfactant is polysorbate. In some embodiments, the polysorbate is polysorbate 20. In some embodiments, the surfactant concentration is from 0.0001% to about 1.0%. In some embodiments, the surfactant concentration is from about 0.01% to about 0.05%. In some embodiments, the surfactant concentration is 0.04%. In some embodiments, the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to 200 mg/ml. In some embodiments, the antibody concentration is from about 30 mg/ml to 175 mg/ml. In some embodiments, the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to about 100 mg/ml. In some embodiments, the antibody is not subject to prior lyophilization. In some embodiments, the antibody binds VEGF. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the monoclonal antibody is a full length antibody. In some embodiments, the monoclonal antibody is an IgG1 antibody. In some embodiments, the monoclonal antibody is a humanized antibody. In some embodiments, the monoclonal antibody is an antibody

fragment comprising an antigen-binding region. In some embodiments, the antibody fragment is a Fab or F(ab')2 fragment. In some embodiments, the monoclonal antibody binds VEGF. In some embodiments, the antibody is bevacizumab. In some embodiments, the monoclonal antibody is susceptible to aggregation. In some embodiments, the buffer is 200 mM arginine acetate pH 5.2, the surfactant is polysorbate in an amount of about 0.01-0.1% v/v and the formulation is stable at a temperature of about 40°C for at least 28 days. In some embodiments, the formulation is sterile. In some embodiments, the formulation is stable upon storage at about 40°C for at least 28 days. In some embodiments, the formulation is aqueous and is administered to a subject. In some embodiments, the formulation is for intravenous (IV), subcutaneous (SQ) or intramuscular (IM) administration. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml.

[0007] Another embodiment of the invention provides an article of manufacture comprising a container holding a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody, an arginine acetate buffer from about pH 4.5 to about 6.0, and a surfactant. In some embodiments, the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to 200 mg/ml. In some embodiments, the antibody concentration is from about 30 mg/ml to 175 mg/ml. In some embodiments, the antibody concentration is from about 50

mg/ml to about 150 mg/ml. In some embodiments, the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to about 100 mg/ml. In some embodiments, the antibody is not subject to prior lyophilization. In some embodiments, the antibody binds VEGF. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the monoclonal antibody is a full length antibody. In some embodiments, the monoclonal antibody is an IgG1 antibody. In some embodiments, the monoclonal antibody is a humanized antibody. In some embodiments, the monoclonal antibody is an antibody fragment comprising an antigen-binding region. In some embodiments, the antibody fragment is a Fab or F(ab')2 fragment. In some embodiments, the monoclonal antibody binds VEGF. In some embodiments, the antibody is bevacizumab. In some embodiments, the monoclonal antibody is susceptible to aggregation. In some embodiments, the arginine actetate concentration in the buffer is from about 25 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 50 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 75 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 100 mM to about 250 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 120 mM to about 240 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 150 mM to about 225 mM. In some embodiments, the arginine acetate concentration in the buffer is about 200 mM. In some embodiments, the arginine acetate buffer has a pH from about 4.5 to about 5.5. In some embodiments, the arginine acetate buffer has a pH from about 4.8 to about 5.4. In some embodiments, the arginine acetate buffer has a pH of about 5.2. In some embodiments, the surfactant is polysorbate. In some embodiments, the polysorbate is polysorbate 20. In some embodiments, the surfactant concentration is from 0.0001% to about 1.0%. In some embodiments, the surfactant concentration is from about 0.01% to about 0.05%. In some embodiments, the surfactant concentration is 0.04%. In some embodiments, the formulation is sterile. In some embodiments, the formulation is stable upon storage at about 40°C for at least 28 days. In some embodiments, the formulation is aqueous and is administered to a subject. In some embodiments, the formulation is for intravenous (IV), subcutaneous (SQ) or intramuscular (IM) administration. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IV administration and the

antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml.

[0008] A further embodiment of the invention provides a method for stabilizing an antibody in an aqueous pharmaceutical formulation by combining a therapeutically effective amount of an antibody, an arginine acetate buffer from about pH 4.5 to about 6.0, and a surfactant. In some embodiments, the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to 200 mg/ml. In some embodiments, the antibody concentration is from about 30 mg/ml to 175 mg/ml. In some embodiments, the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to about 100 mg/ml. In some embodiments, the antibody is not subject to prior lyophilization. In some embodiments, the antibody binds VEGF. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the monoclonal antibody is a full length antibody. In some embodiments, the monoclonal antibody is an IgG1 antibody. In some embodiments, the monoclonal antibody is a humanized antibody. In some embodiments, the monoclonal antibody is an antibody fragment comprising an antigen-binding region. In some embodiments, the antibody fragment is a Fab or F(ab')2 fragment. In some embodiments, the monoclonal antibody binds VEGF. In some embodiments, the antibody is bevacizumab. In some embodiments, the monoclonal antibody is susceptible to aggregation. In some embodiments, the arginine actetate concentration in the buffer is from about 25 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 50 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 75 mM to about 250 mM. In some embodiments, the arginine actetate

concentration in the buffer is from about 100 mM to about 250 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 120 mM to about 240 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 150 mM to about 225 mM. In some embodiments, the arginine acetate concentration in the buffer is about 200 mM. In some embodiments, the arginine acetate buffer has a pH from about 4.5 to about 5.5. In some embodiments, the arginine acetate buffer has a pH from about 4.8 to about 5.4. In some embodiments, the arginine acetate buffer has a pH of about 5.2. In some embodiments, the surfactant is polysorbate. In some embodiments, the polysorbate is polysorbate 20. In some embodiments, the surfactant concentration is from 0.0001% to about 1.0%. In some embodiments, the surfactant concentration is from about 0.01% to about 0.05%. In some embodiments, the surfactant concentration is 0.04%. In some embodiments, the formulation is sterile. In some embodiments, the formulation is stable upon storage at about 40°C for at least 28 days. In some embodiments, the formulation is aqueous and is administered to a subject. In some embodiments, the formulation is for intravenous (IV), subcutaneous (SQ) or intramuscular (IM) administration. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml.

[0009] Yet another embodiment of the invention provides a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody, 200

mM arginine acetate buffer at pH 5.2, and a surfactant. In some embodiments, the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to 200 mg/ml. In some embodiments, the antibody concentration is from about 30 mg/ml to 175 mg/ml. In some embodiments, the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to about 100 mg/ml. In some embodiments, the antibody is not subject to prior lyophilization. In some embodiments, the antibody binds VEGF. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the monoclonal antibody is a full length antibody. In some embodiments, the monoclonal antibody is an IgG1 antibody. In some embodiments, the monoclonal antibody is a humanized antibody. In some embodiments, the monoclonal antibody is an antibody fragment comprising an antigen-binding region. In some embodiments, the antibody fragment is a Fab or F(ab')2 fragment. In some embodiments, the monoclonal antibody binds VEGF. In some embodiments, the antibody is bevacizumab. In some embodiments, the monoclonal antibody is susceptible to aggregation. In some embodiments, the surfactant is polysorbate. In some embodiments, the polysorbate is polysorbate 20. In some embodiments, the surfactant concentration is from 0.0001% to about 1.0%. In some embodiments, the surfactant concentration is from about 0.01% to about 0.05%. In some embodiments, the surfactant concentration is 0.04%. In some embodiments, the formulation is sterile. In some embodiments, the formulation is stable upon storage at about 40°C for at least 28 days. In some embodiments, the formulation is aqueous and is administered to a subject. In some embodiments, the formulation is for intravenous (IV), subcutaneous (SQ) or intramuscular (IM) administration. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from

about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml.

[0010] A further embodiment of the invention provides a pharmaceutical formulation comprising: (a) a full length IgG1 antibody susceptible to deamidation or aggregation in an amount from about 10 mg/mL to about 250 mg/mL; (b) arginine acetate buffer, pH 4.5 to 6.0; and (c) polysorbate 20 in an amount from about 0.01% to about 0.1%. In some embodiments, the antibody concentration is from about 25 mg/ml to 200 mg/ml. In some embodiments, the antibody concentration is from about 30 mg/ml to 175 mg/ml. In some embodiments, the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to about 100 mg/ml. In some embodiments, the antibody is not subject to prior lyophilization. In some embodiments, the antibody binds VEGF. In some embodiments, the antibody is bevacizumab. In some embodiments, the antibody is a humanized antibody. In some embodiments, the arginine actetate concentration in the buffer is from about 25 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 50 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 75 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 100 mM to about 250 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 120 mM to about 240 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 150 mM to about 225 mM. In some embodiments, the arginine acetate concentration in the buffer is about 200 mM. In some embodiments, the arginine acetate buffer has a pH from about 4.5 to about 5.5. In some embodiments, the arginine acetate buffer has a pH from about 4.8 to about 5.4. In some embodiments, the arginine acetate buffer has a pH of about 5.2. In some embodiments, the polysorbate 20 is from about 0.01% to about 0.05%. In some embodiments, the polysorbate 20 is 0.04%. In some embodiments, the formulation is sterile. In some embodiments, the formulation is stable upon storage at about 40°C for at least 28 days. In some embodiments, the formulation is aqueous and is administered to a subject. In some embodiments, the formulation is for intravenous (IV), subcutaneous (SQ) or intramuscular (IM) administration.

In some embodiments, the formulation is for IV administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml.

[0011] Yet another embodiment of the invention provides a pharmaceutical formulation comprising an antibody that binds to VEGF in an arginine acetate buffer at a pH from about 4.5 to about 6.0, and a surfactant. In some embodiments, the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to 200 mg/ml. In some embodiments, the antibody concentration is from about 30 mg/ml to 175 mg/ml. In some embodiments, the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to about 100 mg/ml. In some embodiments, the antibody is not subject to prior lyophilization. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the monoclonal antibody is a full length antibody. In some embodiments, the monoclonal antibody is an IgG1 antibody. In some embodiments, the monoclonal antibody is a humanized antibody. In some embodiments, the monoclonal antibody is an antibody fragment comprising an antigen-binding region. In some embodiments, the antibody fragment is a Fab or F(ab')2 fragment. In some embodiments, the antibody is bevacizumab. In some embodiments, the monoclonal antibody is susceptible to aggregation. In some embodiments, the arginine actetate concentration in the

buffer is from about 25 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 50 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 75 mM to about 250 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 120 mM to about 240 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 150 mM to about 225 mM. In some embodiments, the arginine acetate concentration in the buffer is about 200 mM. In some embodiments, the arginine acetate buffer has a pH from about 4.5 to about 5.5. In some embodiments, the arginine acetate buffer has a pH from about 4.8 to about 5.4. In some embodiments, the arginine acetate buffer has a pH of about 5.2. In some embodiments, the surfactant is polysorbate. In some embodiments, the polysorbate is polysorbate 20. In some embodiments, the surfactant concentration is from 0.0001% to about 1.0%. In some embodiments, the surfactant concentration is from about 0.01% to about 0.05%. In some embodiments, the surfactant concentration is 0.04%. In some embodiments, the formulation is sterile. In some embodiments, the formulation is stable upon storage at about 40°C for at least 28 days. In some embodiments, the formulation is aqueous and is administered to a subject. In some embodiments, the formulation is for intravenous (IV), subcutaneous (SQ) or intramuscular (IM) administration. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml.

[0012] Another embodiment of the invention provides a method for reducing aggregation of a therapeutic monoclonal antibody, comprising formulating the antibody in an arginine acetate buffer, pH 4.5 to 6.0. In some embodiments, the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to 200 mg/ml. In some embodiments, the antibody concentration is from about 30 mg/ml to 175 mg/ml. In some embodiments, the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the antibody concentration is from about 25 mg/ml to about 100 mg/ml. In some embodiments, the antibody is not subject to prior lyophilization. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the monoclonal antibody is a full length antibody. In some embodiments, the monoclonal antibody is an IgG1 antibody. In some embodiments, the monoclonal antibody is a humanized antibody. In some embodiments, the monoclonal antibody is an antibody fragment comprising an antigen-binding region. In some embodiments, the antibody fragment is a Fab or F(ab')2 fragment. In some embodiments, the monoclonal antibody binds VEGF. In some embodiments, the antibody is bevacizumab. In some embodiments, the monoclonal antibody is susceptible to aggregation. In some embodiments, the arginine actetate concentration in the buffer is from about 25 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 50 mM to about 250 mM. In some embodiments, the arginine actetate concentration in the buffer is from about 75 mM to about 250 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 120 mM to about 240 mM. In some embodiments, the arginine acetate concentration in the buffer is from about 150 mM to about 225 mM. In some embodiments, the arginine acetate concentration in the buffer is about 200 mM. In some embodiments, the arginine acetate buffer has a pH from about 4.5 to about 5.5. In some embodiments, the arginine acetate buffer has a pH from about 4.8 to about 5.4. In some embodiments, the arginine acetate buffer has a pH of about 5.2. In some embodiments, the formulation is sterile. In some embodiments, the formulation is stable upon storage at about 40°C for at least 28 days. In some embodiments, the formulation is aqueous and is administered to a subject. In some embodiments, the formulation is for intravenous (IV), subcutaneous (SQ) or intramuscular (IM) administration. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for IV administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IV

administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 25 mg/ml to about 175 mg/ml. In some embodiments, the formulation is for SQ administration and the antibody concentration is from about 50 mg/ml to about 150 mg/ml. In some embodiments, the formulation is for IM administration and the antibody concentration is from about 75 mg/ml to about 125 mg/ml.

[0013] Even a further embodiment of the invention provides an article of manufacture comprising a container holding any one of the formulations described herein.

[0014] Yet a further embodiment of the invention provides a vial with a stopper pierceable by a syringe comprising any one of the formulations described herein. In some embodiments, the vial is stored at about 2-8°C. In some embodiments, the vial is a 3cc, 20 cc or 50 cc vial.

[0015] Another embodiment of the invention provides a stainless steel tank comprising any one of the formulations described herein inside the tank. In some embodiments, the formulation is frozen.

[0016] A further embodiment of the invention provides a method of making a pharmaceutical formulation comprising: (a) preparing any one of the formulations described herein; and (b) evaluating physical stability, chemical stability, or biological activity of the antibody in the formulation.

[0017] Yet another embodiment of the invention provides a method of treating a disease or disorder in a subject comprising administering any one of the formulations described herein to a subject in an amount effective to treat the disease or disorder. In some embodiments, the disease is cancer. In some embodiments, the cancer is selected from colorectal cancer, lung cancer, breast cancer, renal cancer, and glioblastoma.

[0018] These and other embodiments of the invention are further described by the detailed description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1 illustrates total aggregate levels detected in anti-VEGF formulations (100 mg/ml) stored for up to 4 weeks at 40°C.

[0020] Figure 2 illustrates total aggregate levels detected in anti-VEGF formulations (0, 50, 100, and 150 mg/ml).

[0021] Figure 3 illustrates the dimer levels detected in anti-VEGF formulations (100 mg/ml) compared to stored for up to 4 weeks at 40°C.

[0022] Figure 4 illustrates the viscosity of anti-VEGF formulations at 20°C as a function of anti-VEGF concentration (25, 50, 100, 125, 150, or 175 mg/ml).

DETAILED DESCRIPTION

I. Definitions

[0023] Before describing the invention in detail, it is to be understood that this invention is not limited to particular compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a molecule" optionally includes a combination of two or more such molecules, and the like.

[0024] The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of the active ingredient to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. Such formulations are sterile. "Pharmaceutically acceptable" excipients (vehicles, additives) are those which can reasonably be administered to a subject mammal to provide an effective dose of the active ingredient employed.

[0025] A "sterile" formulation is asceptic or free or essentially free from all living microorganisms and their spores.

[0026] Herein, a "frozen" formulation is one at a temperature below 0oC. Generally, the frozen formulation is not freeze-dried, nor is it subjected to prior, or subsequent, lyophilization. In certain embodiments, the frozen formulation comprises frozen drug substance for storage (in stainless steel tank) or frozen drug product (in final vial configuration).

[0027] A "stable" formulation is one in which the protein therein essentially retains its physical stability and/or chemical stability and/or biological activity upon storage. Preferably, the formulation essentially retains its physical and chemical stability, as well as its biological

activity upon storage. The storage period is generally selected based on the intended shelf-life of the formulation. Various analytical techniques for measuring protein stability are available in the art and are reviewed in *Peptide and Protein Drug Delivery*, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. *Adv. Drug Delivery Rev.* 10: 29-90 (1993), for example. Stability can be measured at a selected temperature for a selected time period. In certain embodiments, the formulation is stable at about 40°C for at least about 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, or more days. In certain embodiments, the formulation is stable at about 40°C for at least about 1, 2, 3, 4, 5, 6, 7, 8, or more weeks. In certain embodiments, the formulation is stable at about 25°C for at least 1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or more months. In certain embodiments, the formulation is stable at about 5°C for at least 1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or more months. In certain embodiments, the formulation is stable at about -20°C for at least 1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, or more months. In certain embodiments, the formulation is stable at 5°C or -20°C for at least 1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, or more months. Furthermore, the formulation is preferably stable following freezing (to, e.g., -20°C or -70°C) and thawing of the formulation, for example following 1, 2 3, 4, or 5 cycles of freezing and thawing. Stability can be evaluated qualitatively and/or quantitatively in a variety of different ways, including evaluation of aggregate formation (for example using size exclusion chromatography, by measuring turbidity, and/or by visual inspection); by assessing charge heterogeneity using cation exchange chromatography, image capillary isoelectric focusing (icIEF) or capillary zone electrophoresis; amino-terminal or carboxy-terminal sequence analysis; mass spectrometric analysis; SDS-PAGE analysis to compare reduced and intact antibody; peptide map (for example tryptic or LYS-C) analysis; evaluating biological activity or antigen binding function of the antibody; etc. Instability may involve any one or more of: aggregation, deamidation (e.g. Asn deamidation), oxidation (e.g. Met oxidation), isomerization (e.g. Asp isomeriation), clipping/hydrolysis/fragmentation (e.g. hinge region fragmentation), succinimide formation, unpaired cysteine(s), N-terminal extension, C-terminal processing, glycosylation differences, etc.

[0028] A protein "retains its physical stability" in a pharmaceutical formulation if it shows no signs or very little of aggregation, precipitation and/or denaturation upon visual examination of color and/or clarity, or as measured by UV light scattering or by size exclusion chromatography.

[0029] A protein "retains its chemical stability" in a pharmaceutical formulation, if the chemical stability at a given time is such that the protein is considered to still retain its biological activity as defined below. Chemical stability can be assessed by detecting and quantifying chemically altered forms of the protein. Chemical alteration may involve size modification (e.g. clipping) which can be evaluated using size exclusion chromatography, SDS-PAGE and/or matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI/TOF MS), for example. Other types of chemical alteration include charge alteration (e.g. occurring as a result of deamidation) which can be evaluated by ion-exchange chromatography or icIEF, for example.

[0030] An antibody "retains its biological activity" in a pharmaceutical formulation, if the biological activity of the antibody at a given time is within about 10% (within the errors of the assay) of the biological activity exhibited at the time the pharmaceutical formulation was prepared as determined in an antigen binding assay, for example. Other "biological activity" assays for antibodies are elaborated herein below.

[0031] Herein, "biological activity" of a monoclonal antibody refers to the ability of the antibody to bind to antigen. It can further include antibody binding to antigen and resulting in a measurable biological response which can be measured in vitro or in vivo. Such activity may be antagonistic or agonistic.

[0032] A "deamidated" monoclonal antibody herein is one in which one or more asparagine residue thereof has been derivitized, e.g. to an aspartic acid or an iso-aspartic acid.

[0033] An antibody which is "susceptible to deamidation" is one comprising one or more residue which has been found to be prone to deamidate.

[0034] An antibody which is "susceptible to aggregation" is one which has been found to aggregate with other antibody molecule(s), especially upon freezing and/or agitation.

[0035] An antibody which is "susceptible to fragmentation" is one which has been found to be cleaved into two or more fragments, for example at a hinge region thereof.

[0036] By "reducing deamidation, aggregation, or fragmentation" is intended preventing or decreasing the amount of deamidation, aggregation, or fragmentation relative to the monoclonal antibody formulated at a different pH or in a different buffer.

[0037] The antibody which is formulated is preferably essentially pure and desirably essentially homogeneous (e.g., free from contaminating proteins etc). "Essentially pure" antibody means a composition comprising at least about 90% by weight of the antibody, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" antibody means a composition comprising at least about 99% by weight of antibody, based on total weight of the composition.

[0038] By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

[0039] As used herein, "buffer" refers to a buffered solution that resists changes in pH by the action of its acid-base conjugate components. The buffer of this invention preferably has a pH in the range from about 4.5 to about 7.0, preferably from about 4.5 to about 6.5, for example from 4.5 to 6.0, 4.5 to 5.9. 4.5 to 5.8, 4.5 to 5.7, 4.5 to 5.6, 4.5 to 5.5, 4.5 to 5.6, 4.5 to 5.5, 4.5 to 5.4, 4.5 to 5.3, 4.5 to 5.2, 4.5 to 5.1, 4.5 to 5.0, 4.5 to 4.9, 4.5 to 4.8, 4.5 to 4.7, or 4.5 to 4.6. In one embodiment the buffer has a pH 4.5, 4.6, 4.7, 4.8, 4.8, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, or 6.0. Examples of buffers that will control the pH in this range include acetate, succinate, succinate, gluconate, histidine, citrate, glycylglycine and other organic acid buffers.

[0040] An "arginine buffer" is a buffer comprising arginine ions. Examples of arginine buffers include arginine acetate, arginine chloride, arginine phosphate, arginine sulfate, arginine succinate, etc. In one embodiment, the arginine buffer is arginine acetate. In the one embodiment, the arginine acetate buffer is prepared by titrating L- arginine (free base, solid) with acetic acid (liquid). In certain embodiments, the arginine buffer is at pH 4.5 to 6.0, 4.5 to 5.9. 4.5 to 5.8, 4.5 to 5.7, 4.5 to 5.6, 4.5 to 5.5, 4.5 to 5.6, 4.5 to 5.5, 4.5 to 5.4, 4.5 to 5.3, 4.5 to 5.2, 4.5 to 5.1, 4.5 to 5.0, 4.5 to 4.9, 4.5 to 4.8, 4.5 to 4.7, or 4.5 to 4.6. In one embodiment the buffer has a pH 4.5, 4.6, 4.7, 4.8, 4.8, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, or 6.0.

[0041] Herein, a "surfactant" refers to a surface-active agent, preferably a nonionic surfactant. Examples of surfactants herein include polysorbate (for example, polysorbate 20 and, polysorbate 80); poloxamer (e.g. poloxamer 188); Triton; sodium dodecyl sulfate (SDS); sodium laurel sulfate; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-betaine (e.g. lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate; and the MONAQUAT™ series (Mona Industries, Inc., Paterson, N.J.); polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (e.g. Pluronics, PF68 etc); etc. In one embodiment, the surfactant herein is polysorbate 20.

[0042] In a pharmacological sense, in the context of the invention, a "therapeutically effective amount" of an antibody refers to an amount effective in the prevention or treatment of a disorder for the treatment of which the antibody is effective. A "disorder" is any condition

that would benefit from treatment with the antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

[0043] A "preservative" is a compound which can be optionally included in the formulation to essentially reduce bacterial action therein, thus facilitating the production of a multi-use formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. In one embodiment, the preservative herein is benzyl alcohol.

[0044] A "polyol" is a substance with multiple hydroxyl groups, and includes sugars (reducing and nonreducing sugars), sugar alcohols and sugar acids. A polyol may optionally be included in the formulation. In certain embodiments, polyols herein have a molecular weight which is less than about 600 kD (e.g. in the range from about 120 to about 400 kD). A "reducing sugar" is one which contains a hemiacetal group that can reduce metal ions or react covalently with lysine and other amino groups in proteins and a "nonreducing sugar" is one which does not have these properties of a reducing sugar. Examples of reducing sugars are fructose, mannose, maltose, lactose, arabinose, xylose, ribose, rhamnose, galactose and glucose. Nonreducing sugars include sucrose, trehalose, sorbose, melezitose and raffinose. Mannitol, xylitol, erythritol, threitol, sorbitol and glycerol are examples of sugar alcohols. As to sugar acids, these include L-gluconate and metallic salts thereof. Where it desired that the formulation is freeze-thaw stable, the polyol is preferably one which does not crystallize at freezing temperatures (e.g. -20^{0}C) such that it destabilizes the antibody in the formulation. In certain embodiments, nonreducing sugars such as sucrose and trehalose are examples of polyols, with trehalose being preferred over sucrose, because of the solution stability of trehalose.

[0045] The term "VEGF" or "VEGF-A" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor and related 121-, 189-, and 206- amino acid human vascular endothelial cell growth factors, as described by Leung et al. (1989) *Science* 246:1306, and Houck et al. (1991) *Mol. Endocrin*, 5:1806, together with the naturally occurring allelic and processed forms thereof. The term "VEGF" also refers to VEGFs from non-human species such as mouse, rat or primate. Sometimes the VEGF from a specific species are indicated by terms such as hVEGF for human VEGF, mVEGF for murine VEGF,

and etc. The term "VEGF" is also used to refer to truncated forms of the polypeptide comprising amino acids 8 to 109 or 1 to 109 of the 165-amino acid human vascular endothelial cell growth factor. Reference to any such forms of VEGF may be identified in the present application, e.g., by "VEGF (8-109)," "VEGF (1-109)" or "$VEGF_{165}$." The amino acid positions for a "truncated" native VEGF are numbered as indicated in the native VEGF sequence. For example, amino acid position 17 (methionine) in truncated native VEGF is also position 17 (methionine) in native VEGF. The truncated native VEGF has binding affinity for the KDR and Flt-1 receptors comparable to native VEGF.

[0046] "VEGF biological activity" includes binding to any VEGF receptor or any VEGF signaling activity such as regulation of both normal and abnormal angiogenesis and vasculogenesis (Ferrara and Davis-Smyth (1997) *Endocrine Rev.* 18:4-25; Ferrara (1999) *J. Mol. Med.* 77:527-543); promoting embryonic vasculogenesis and angiogenesis (Carmeliet et al. (1996) *Nature* 380:435-439; Ferrara et al. (1996) *Nature* 380:439-442); and modulating the cyclical blood vessel proliferation in the female reproductive tract and for bone growth and cartilage formation (Ferrara et al. (1998) *Nature Med.* 4:336-340; Gerber et al. (1999) *Nature Med.* 5:623-628). In addition to being an angiogenic factor in angiogenesis and vasculogenesis, VEGF, as a pleiotropic growth factor, exhibits multiple biological effects in other physiological processes, such as endothelial cell survival, vessel permeability and vasodilation, monocyte chemotaxis and calcium influx (Ferrara and Davis-Smyth (1997), *supra* and Cebe-Suarez et al. *Cell. Mol. Life Sci.* 63:601-615 (2006)). Moreover, recent studies have reported mitogenic effects of VEGF on a few non-endothelial cell types, such as retinal pigment epithelial cells, pancreatic duct cells, and Schwann cells. Guerrin et al. (1995) *J. Cell Physiol.* 164:385-394; Oberg-Welsh et al. (1997) *Mol. Cell. Endocrinol.* 126:125-132; Sondell et al. (1999) *J. Neurosci.* 19:5731-5740.

[0047] A "VEGF antagonist" or "VEGF-specific antagonist" refers to a molecule capable of binding to VEGF, reducing VEGF expression levels, or neutralizing, blocking, inhibiting, abrogating, reducing, or interfering with VEGF biological activities, including, but not limited to, VEGF binding to one or more VEGF receptors and VEGF mediated angiogenesis and endothelial cell survival or proliferation. Included as VEGF-specific antagonists useful in the methods of the invention are polypeptides that specifically bind to VEGF, anti-VEGF antibodies and antigen-binding fragments thereof, receptor molecules and derivatives which bind specifically to VEGF thereby sequestering its binding to one or more receptors, fusions proteins (e.g., VEGF-Trap (Regeneron)), and $VEGF_{121}$-gelonin (Peregrine). VEGF-specific antagonists also include antagonist variants of VEGF polypeptides, antisense nucleobase oligomers directed to VEGF, small RNA molecules directed to VEGF, RNA

aptamers, peptibodies, and ribozymes against VEGF. VEGF-specific antagonists also include nonpeptide small molecules that bind to VEGF and are capable of blocking, inhibiting, abrogating, reducing, or interfering with VEGF biological activities. Thus, the term "VEGF activities" specifically includes VEGF mediated biological activities of VEGF. In certain embodiments, the VEGF antagonist reduces or inhibits, by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more, the expression level or biological activity of VEGF.

[0048] An "anti-VEGF antibody" is an antibody that binds to VEGF with sufficient affinity and specificity. In certain embodiments, the antibody selected will normally have a sufficiently binding affinity for VEGF, for example, the antibody may bind hVEGF with a K_d value of between 100 nM-1 pM. Antibody affinities may be determined by a surface plasmon resonance based assay (such as the BIAcore assay as described in PCT Application Publication No. WO2005/012359); enzyme-linked immunoabsorbent assay (ELISA); and competition assays (e.g. RIA's), for example.

[0049] In certain embodiment, the anti-VEGF antibody can be used as a therapeutic agent in targeting and interfering with diseases or conditions wherein the VEGF activity is involved. Also, the antibody may be subjected to other biological activity assays, e.g., in order to evaluate its effectiveness as a therapeutic. Such assays are known in the art and depend on the target antigen and intended use for the antibody. Examples include the HUVEC inhibition assay; tumor cell growth inhibition assays (as described in WO 89/06692, for example); antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC) assays (US Patent 5,500,362); and agonistic activity or hematopoiesis assays (see WO 95/27062). An anti-VEGF antibody will usually not bind to other VEGF homologues such as VEGF-B or VEGF-C, nor other growth factors such as PlGF, PDGF or bFGF. In one embodiment, anti-VEGF antibody is a monoclonal antibody that binds to the same epitope as the monoclonal anti-VEGF antibody A4.6.1 produced by hybridoma ATCC HB 10709. In another embodiment, the anti-VEGF antibody is a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. (1997) *Cancer Res.* 57:4593-4599, including but not limited to the antibody known as bevacizumab (BV; AVASTIN®).

[0050] The anti-VEGF antibody "Bevacizumab (BV)," also known as "rhuMAb VEGF" or "AVASTIN®," is a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. (1997) *Cancer Res.* 57:4593-4599. It comprises mutated human IgG1 framework regions and antigen-binding complementarity-determining regions from the murine anti-hVEGF monoclonal antibody A.4.6.1 that blocks binding of human VEGF to its receptors. Approximately 93% of the amino acid sequence of Bevacizumab, including most of the framework regions, is derived from human IgG1, and about 7% of the

sequence is derived from the murine antibody A4.6.1. Bevacizumab has a molecular mass of about 149,000 daltons and is glycosylated. Bevacizumab and other humanized anti-VEGF antibodies are further described in U.S. Pat. No. 6,884,879 issued Feb. 26, 2005, the entire disclosure of which is expressly incorporated herein by reference.

[0051] The term "B20 series polypeptide" as used herein refers to a polypeptide, including an antibody that binds to VEGF. B20 series polypeptides includes, but not limited to, antibodies derived from a sequence of the B20 antibody or a B20-derived antibody described in US Publication No. 20060280747, US Publication No. 20070141065 and/or US Publication No. 20070020267, the content of these patent applications are expressly incorporated herein by reference. In one embodiment, B20 series polypeptide is B20-4.1 as described in US Publication No. 20060280747, US Publication No. 20070141065 and/or US Publication No. 20070020267. In another embodiment, B20 series polypeptide is B20-4.1.1 described in US Patent Application 60/991,302, the entire disclosure of which is expressly incorporated herein by reference.

[0052] The term "G6 series polypeptide" as used herein refers to a polypeptide, including an antibody that binds to VEGF. G6 series polypeptides includes, but not limited to, antibodies derived from a sequence of the G6 antibody or a G6-derived antibody described in US Publication No. 20060280747, US Publication No. 20070141065 and/or US Publication No. 20070020267. G6 series polypeptides, as described in US Publication No. 20060280747, US Publication No. 20070141065 and/or US Publication No. 20070020267 include, but not limited to, G6-8, G6-23 and G6-31.

[0053] For additional antibodies *see* U.S. Pat. Nos. 7,060,269, 6,582,959, 6,703,020; 6,054,297; WO98/45332; WO 96/30046; WO94/10202; EP 0666868B1; U.S. Patent Application Publication Nos. 2006009360, 20050186208, 20030206899, 20030190317, 20030203409, and 20050112126; and Popkov *et al., Journal of Immunological Methods* 288:149-164 (2004). In certain embodiments, other antibodies include those that bind to a functional epitope on human VEGF comprising of residues F17, M18, D19, Y21, Y25, Q89, I91, K101, E103, and C104 or, alternatively, comprising residues F17, Y21, Q22, Y25, D63, I83 and Q89.

[0054] Other anti-VEGF antibodies are also known, and described, for example, in Liang et al., *J Biol Chem* 281, 951-961 (2006).

[0055] "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

[0056] A "disorder" is any condition that would benefit from treatment including, but not limited to, chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Disorders include angiogenic disorders. "Angiogenic disorder" as used herein refers to any condition involving abnormal angiogenesis or abnormal vascular permeability or leakage. Non-limiting examples of angiogenic disorders to be treated herein include malignant and benign tumors; non-leukemias and lymphoid malignancies; and, in particular, tumor (cancer) metastasis.

[0057] "Abnormal angiogenesis" occurs when new blood vessels grow either excessively or otherwise inappropriately (e.g., the location, timing, degree, or onset of the angiogenesis being undesired from a medical standpoint) in a diseased state or such that it causes a diseased state. In some cases, excessive, uncontrolled, or otherwise inappropriate angiogenesis occurs when there is new blood vessel growth that contributes to the worsening of the diseased state or cause of a diseased state. The new blood vessels can feed the diseased tissues, destroy normal tissues, and in the case of cancer, the new vessels can allow tumor cells to escape into the circulation and lodge in other organs (tumor metastases). Examples of disorders involving abnormal angiogenesis include, but are not limited to cancer, especially vascularized solid tumors and metastatic tumors (including colon, lung cancer (especially small-cell lung cancer), or prostate cancer), diseases caused by ocular neovascularisation, especially diabetic blindness, retinopathies, primarily diabetic retinopathy or age-related macular degeneration, choroidal neovascularization (CNV), diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, Central Retinal Vein Occlusion (CRVO), corneal neovascularization, retinal neovascularization and rubeosis; psoriasis, psoriatic arthritis, haemangioblastoma such as haemangioma; inflammatory renal diseases, such as glomerulonephritis, especially mesangioproliferative glomerulonephritis, haemolytic uremic syndrome, diabetic nephropathy or hypertensive nephrosclerosis; various imflammatory diseases, such as arthritis, especially rheumatoid arthritis, inflammatory bowel disease, psorsasis, sarcoidosis, arterial arteriosclerosis and diseases occurring after transplants, endometriosis or chronic asthma and other conditions.

[0058] "Abnormal vascular permeability" occurs when the flow of fluids, molecules (e.g., ions and nutrients) and cells (e.g., lymphocytes) between the vascular and extravascular compartments is excessive or otherwise inappropriate (*e.g.*, the location, timing, degree, or onset of the vascular permeability being undesired from a medical standpoint) in a diseased state or such that it causes a diseased state. Abnormal vascular permeability may lead to excessive or otherwise inappropriate "leakage" of ions, water, nutrients, or cells through the vasculature. In some cases, excessive, uncontrolled, or otherwise inappropriate vascular

permeability or vascular leakage exacerbates or induces disease states including, e.g., edema associated with tumors including, e.g., brain tumors; ascites associated with malignancies; Meigs' syndrome; lung inflammation; nephrotic syndrome; pericardial effusion; pleural effusion,; permeability associated with cardiovascular diseases such as the condition following myocardial infarctions and strokes and the like. The present invention contemplates treating those patients that have developed or are at risk of developing the diseases and disorders associated with abnormal vascular permeability or leakage.

[0059] The terms "cell proliferative disorder" and "proliferative disorder" refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer. In one embodiment, the cell proliferative disorder is a tumor.

[0060] "Tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "cancer", "cancerous", "cell proliferative disorder", "proliferative disorder" and "tumor" are not mutually exclusive as referred to herein.

[0061] The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include, but not limited to, squamous cell cancer (*e.g.*, epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer and gastrointestinal stromal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, cancer of the urinary tract, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, melanoma, superficial spreading melanoma, lentigo maligna melanoma, acral lentiginous melanomas, nodular melanomas, multiple myeloma and B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder

(PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), Meigs' syndrome, brain, as well as head and neck cancer, and associated metastases. In certain embodiments, cancers that are amenable to treatment by the antibodies of the invention include breast cancer, colorectal cancer, rectal cancer, non-small cell lung cancer, glioblastoma, non-Hodgkins lymphoma (NHL), renal cell cancer, prostate cancer, liver cancer, pancreatic cancer, soft-tissue sarcoma, kaposi's sarcoma, carcinoid carcinoma, head and neck cancer, ovarian cancer, mesothelioma, and multiple myeloma. In some embodiments, the cancer is selected from: small cell lung cancer, gliblastoma, neuroblastomas, melanoma, breast carcinoma, gastric cancer, colorectal cancer (CRC), and hepatocellular carcinoma. Yet, in some embodiments, the cancer is selected from: non-small cell lung cancer, colorectal cancer, glioblastoma and breast carcinoma, including metastatic forms of those cancers.

[0062] The term "anti-cancer therapy" refers to a therapy useful in treating cancer. Examples of anti-cancer therapeutic agents include, but are limited to, e.g., chemotherapeutic agents, growth inhibitory agents, cytotoxic agents, agents used in radiation therapy, anti-angiogenic agents, apoptotic agents, anti-tubulin agents, and other agents to treat cancer, such as anti-HER-2 antibodies, anti-CD20 antibodies, an epidermal growth factor receptor (EGFR) antagonist (e.g., a tyrosine kinase inhibitor), HER1/EGFR inhibitor (e.g., erlotinib (Tarceva™), platelet derived growth factor inhibitors (e.g., Gleevec™ (Imatinib Mesylate)), a COX-2 inhibitor (e.g., celecoxib), interferons, cytokines, antagonists (e.g., neutralizing antibodies) that bind to one or more of the following targets ErbB2, ErbB3, ErbB4, PDGFR-beta, BlyS, APRIL, BCMA or VEGF receptor(s), TRAIL/Apo2, and other bioactive and organic chemical agents, etc. Combinations thereof are also included in the invention.

[0063] An "angiogenic factor or agent" is a growth factor or its receptor which is involved in stimulating the development of blood vessels, *e.g.*, promote angiogenesis, endothelial cell growth, stabiliy of blood vessels, and/or vasculogenesis, *etc.* For example, angiogenic factors, include, but are not limited to, *e.g.*, VEGF and members of the VEGF family and their receptors (VEGF-B, VEGF-C, VEGF-D, VEGFR1, VEGFR2 and VEGFR3), PlGF, PDGF family, fibroblast growth factor family (FGFs), TIE ligands (Angiopoietins, ANGPT1, ANGPT2), TIE1, TIE2, ephrins, Bv8, Delta-like ligand 4 (DLL4), Del-1, fibroblast growth factors: acidic (aFGF) and basic (bFGF), FGF4, FGF9, BMP9, BMP10, Follistatin, Granulocyte colony-stimulating factor (G-CSF), GM-CSF, Hepatocyte growth factor (HGF) /scatter factor (SF), Interleukin-8 (IL-8), CXCL12, Leptin, Midkine, neuropilins, NRP1, NRP2, Placental growth factor, Platelet-derived endothelial cell growth factor (PD-ECGF), Platelet-derived growth factor, especially PDGF-BB, PDGFR-alpha, or PDGFR-beta, Pleiotrophin

(PTN), Progranulin, Proliferin, Transforming growth factor-alpha (TGF-alpha), Transforming growth factor-beta (TGF-beta), Tumor necrosis factor-alpha (TNF-alpha), Alk1, CXCR4, Notch1, Notch4, Sema3A, Sema3C, Sema3F, Robo4, *etc.* It would further include factors that promote angiogenesis, such as ESM1 and Perlecan. It would also include factors that accelerate wound healing, such as growth hormone, insulin-like growth factor-I (IGF-I), VIGF, epidermal growth factor (EGF), EGF-like domain, multiple 7 (EGFL7), CTGF and members of its family, and TGF-alpha and TGF-beta. *See, e.g.,* Klagsbrun and D'Amore (1991) *Annu. Rev. Physiol.* 53:217-39; Streit and Detmar (2003) *Oncogene* 22:3172-3179; Ferrara & Alitalo (1999) *Nature Medicine* 5(12):1359-1364; Tonini et al. (2003) *Oncogene* 22:6549-6556 (e.g., Table 1 listing known angiogenic factors); and, Sato (2003) *Int. J. Clin. Oncol.* 8:200-206.

[0064] An "anti-angiogenic agent" or "angiogenic inhibitor" refers to a small molecular weight substance, a polynucleotide (including, e.g., an inhibitory RNA (RNAi or siRNA)), a polypeptide, an isolated protein, a recombinant protein, an antibody, or conjugates or fusion proteins thereof, that inhibits angiogenesis, vasculogenesis, or undesirable vascular permeability, either directly or indirectly. It should be understood that the anti-angiogenic agent includes those agents that bind and block the angiogenic activity of the angiogenic factor or its receptor. For example, an anti-angiogenic agent is an antibody or other antagonist to an angiogenic agent as defined above, e.g., antibodies to VEGF-A or to the VEGF-A receptor (e.g., KDR receptor or Flt-1 receptor), anti-PDGFR inhibitors, small molecules that block VEGF receptor signaling (e.g., PTK787/ZK2284, SU6668, SUTENT®/SU11248 (sunitinib malate), AMG706, or those described in, e.g., international patent application WO 2004/113304). Anti-angiogenic agents include, but are not limited to, the following agents: VEGF inhibitors such as a VEGF-specific antagonist, EGF inhibitor, EGFR inhibitors, Erbitux® (cetuximab, ImClone Systems, Inc., Branchburg, N.J.), Vectibix® (panitumumab, Amgen, Thousand Oaks, CA), TIE2 inhibitors, IGF1R inhibitors, COX-II (cyclooxygenase II) inhibitors, MMP-2 (matrix-metalloproteinase 2) inhibitors, and MMP-9 (matrix-metalloproteinase 9) inhibitors, CP-547,632 (Pfizer Inc., NY, USA), Axitinib (Pfizer Inc.; AG-013736), ZD-6474 (AstraZeneca), AEE788 (Novartis), AZD-2171), VEGF Trap (Regeneron/Aventis), Vatalanib (also known as PTK-787, ZK-222584: Novartis & Schering A G), Macugen (pegaptanib octasodium, NX-1838, EYE-001, Pfizer Inc./Gilead/Eyetech), IM862 (Cytran Inc. of Kirkland, Wash., USA); and angiozyme, a synthetic ribozyme from Ribozyme (Boulder, Colo.) and Chiron (Emeryville, Calif.) and combinations thereof. Other angiogenesis inhibitors include thrombospondin1, thrombospondin2, collagen IV and collagen XVIII. VEGF inhibitors are disclosed in U.S. Pat. Nos. 6,534,524 and 6,235,764, both of which are incorporated in their entirety for all purposes. Anti-angiogenic agents also include

native angiogenesis inhibitors , e.g., angiostatin, endostatin, etc. *See, e.g.,* Klagsbrun and D'Amore (1991) *Annu. Rev. Physiol.* 53:217-39; Streit and Detmar (2003) *Oncogene* 22:3172-3179 (e.g., Table 3 listing anti-angiogenic therapy in malignant melanoma); Ferrara & Alitalo (1999) *Nature Medicine* 5(12):1359-1364; Tonini et al. (2003) *Oncogene* 22:6549-6556 (e.g., Table 2 listing known antiangiogenic factors); and, Sato (2003) *Int. J. Clin. Oncol.* 8:200-206 (e.g., Table 1 listing anti-angiogenic agents used in clinical trials).

[0065] The term "anti-angiogenic therapy" refers to a therapy useful for inhibiting angiogenesis which comprises the administration of an anti-angiogenic agent.

[0066] The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. The term is intended to include radioactive isotopes (e.g., At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu), chemotherapeutic agents (e.g., methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

[0067] A "toxin" is any substance capable of having a detrimental effect on the growth or proliferation of a cell.

[0068] A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN®); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, chlorophosphamide, estramustine,

ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, e.g., Nicolaou *et al.*, *Angew. Chem Intl. Ed. Engl.*, 33: 183-186 (1994)); CDP323, an oral alpha-4 integrin inhibitor; dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection (DOXIL®), liposomal doxorubicin TLC D-99 (MYOCET®), peglylated liposomal doxorubicin (CAELYX®), and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, and 5-fluorouracil (5-FU); combretastatin; folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2'-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoid, *e.g.*, paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.), albumin-engineered nanoparticle

formulation of paclitaxel (ABRAXANE™), and docetaxel (TAXOTERE®, Rhône-Poulene Rorer, Antony, France); chloranbucil; 6-thioguanine; mercaptopurine; methotrexate; platinum agents such as cisplatin, oxaliplatin (e.g., ELOXATIN®), and carboplatin; vincas, which prevent tubulin polymerization from forming microtubules, including vinblastine (VELBAN®), vincristine (ONCOVIN®), vindesine (ELDISINE®, FILDESIN®), and vinorelbine (NAVELBINE®); etoposide (VP-16); ifosfamide; mitoxantrone; leucovorin; novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid, including bexarotene (TARGRETIN®); bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), etidronate (DIDROCAL®), NE-58095, zoledronic acid/zoledronate (ZOMETA®), alendronate (FOSAMAX®), pamidronate (AREDIA®), tiludronate (SKELID®), or risedronate (ACTONEL®); troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R) (e.g., erlotinib (Tarceva™)); and VEGF-A that reduce cell proliferation; vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; topoisomerase 1 inhibitor (*e.g.*, LURTOTECAN®); rmRH (*e.g.*, ABARELIX®); BAY439006 (sorafenib; Bayer); SU-11248 (sunitinib, SUTENT®, Pfizer); perifosine, COX-2 inhibitor (*e.g.* celecoxib or etoricoxib), proteosome inhibitor (*e.g.* PS341); bortezomib (VELCADE®); CCI-779; tipifarnib (R11577); orafenib, ABT510; Bcl-2 inhibitor such as oblimersen sodium (GENASENSE®); pixantrone; EGFR inhibitors; tyrosine kinase inhibitors; serine-threonine kinase inhibitors such as rapamycin (sirolimus, RAPAMUNE®); farnesyltransferase inhibitors such as lonafarnib (SCH 6636, SARASAR™); and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone; and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovorin, and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above.

[0069] Chemotherapeutic agents as defined herein include "anti-hormonal agents" or "endocrine therapeutics" which act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer. They may be hormones themselves, including, but not limited to: anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen),

raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON· toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf and H-Ras; ribozymes such as a VEGF expression inhibitor (e.g., ANGIOZYME® ribozyme) and a HER2 expression inhibitor; vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; Vinorelbine and Esperamicins (see U.S. Pat. No. 4,675,187), and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above.

[0070] A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell either *in vitro* or *in vivo*. In one embodiment, growth inhibitory agent is growth inhibitory antibody that prevents or reduces proliferation of a cell expressing an antigen to which the antibody binds. In another embodiment, the growth inhibitory agent may be one which significantly reduces the percentage of cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in Mendelsohn and Israel, eds., *The Molecular Basis of Cancer*, Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (W.B. Saunders, Philadelphia, 1995), *e.g.*, p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

[0071] By "radiation therapy" is meant the use of directed gamma rays or beta rays to induce sufficient damage to a cell so as to limit its ability to function normally or to destroy the cell altogether. It will be appreciated that there will be many ways known in the art to determine the dosage and duration of treatment. Typical treatments are given as a one time administration and typical dosages range from 10 to 200 units (Grays) per day.

[0072] "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

[0073] The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

[0074] An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

[0075] "Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain.

Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

[0076] The term "constant domain" refers to the portion of an immunoglobulin molecule having a more conserved amino acid sequence relative to the other portion of the immunoglobulin, the variable domain, which contains the antigen binding site. The constant domain contains the C_H1, C_H2 and C_H3 domains (collectively, CH) of the heavy chain and the CHL (or CL) domain of the light chain.

[0077] The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "VH." The variable domain of the light chain may be referred to as "VL." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

[0078] The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three HVRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The HVRs in each chain are held together in close proximity by the FR regions and, with the HVRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in the binding of an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

[0079] The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

[0080] The term IgG "isotype: or "subclass" as used herein is meant any of the subclasses of immunoglobulins defined by the chemical and antigenic characteristics of their constant regions.

[0081] Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five

major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al. *Cellular and Mol. Immunology*, 4th ed. (W.B. Saunders, Co., 2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

[0082] The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain an Fc region.

[0083] A "naked antibody" for the purposes herein is an antibody that is not conjugated to a cytotoxic moiety or radiolabel.

[0084] "Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

[0085] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

[0086] "Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three HVRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six HVRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0087] The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0088] "Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see, *e.g.*, Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York, 1994), pp. 269-315.

[0089] The term "diabodies" refers to antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO 1993/01161; Hudson et al., *Nat. Med.* 9:129-134 (2003); and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., *Nat. Med.* 9:129-134 (2003).

[0090] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, e.g., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. In certain embodiments, such a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to

humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity *in vivo*, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

[0091] The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler and Milstein, *Nature*, 256:495-97 (1975); Hongo *et al.*, *Hybridoma*, 14 (3): 253-260 (1995), Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567), phage-display technologies (see, e.g., Clackson *et al.*, *Nature*, 352: 624-628 (1991); Marks *et al.*, *J. Mol. Biol.* 222: 581-597 (1992); Sidhu *et al.*, *J. Mol. Biol.* 338(2): 299-310 (2004); Lee *et al.*, *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee *et al.*, *J. Immunol. Methods* 284(1-2): 119-132(2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits *et al.*, *Nature* 362: 255-258 (1993); Bruggemann *et al.*, *Year in Immunol.* 7:33 (1993); U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks *et al.*, *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild *et al.*, *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

[0092] The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or

belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (see, *e.g.*,U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Chimeric antibodies include PRIMATIZED® antibodies wherein the antigen-binding region of the antibody is derived from an antibody produced by, e.g., immunizing macaque monkeys with the antigen of interest.

[0093] "Humanized" forms of non-human (*e.g.,* murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a HVR of the recipient are replaced by residues from a HVR of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, FR residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, *e.g.*, Jones *et al., Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also, *e.g.*, Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409.

[0094] A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries. Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581 (1991). Also available for the preparation of human monoclonal antibodies are methods described in Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner *et al.*, *J. Immunol.*, 147(1):86-95 (1991). See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.,* 5: 368-74 (2001). Human

antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, *e.g.*, immunized xenomice (see, *e.g.*, U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li *et al., Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology.

[0095] A "species-dependent antibody" is one which has a stronger binding affinity for an antigen from a first mammalian species than it has for a homologue of that antigen from a second mammalian species. Normally, the species-dependent antibody "binds specifically" to a human antigen (e.g., has a binding affinity (Kd) value of no more than about 1 x 10-7 M, preferably no more than about 1 x 10-8 M and preferably no more than about 1 x 10-9 M) but has a binding affinity for a homologue of the antigen from a second nonhuman mammalian species which is at least about 50 fold, or at least about 500 fold, or at least about 1000 fold, weaker than its binding affinity for the human antigen. The species-dependent antibody can be any of the various types of antibodies as defined above, but preferably is a humanized or human antibody.

[0096] The term "hypervariable region," "HVR," or "HV," when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, *e.g.*, Xu et al., *Immunity* 13:37-45 (2000); Johnson and Wu, in *Methods in Molecular Biology* 248:1-25 (Lo, ed., Human Press, Totowa, NJ, 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain. See, *e.g.*, Hamers-Casterman et al., *Nature* 363:446-448 (1993); Sheriff et al., *Nature Struct. Biol.* 3:733-736 (1996).

[0097] A number of HVR delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). The AbM HVRs represent a compromise between the Kabat HVRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" HVRs are based on an analysis of the available complex crystal structures. The residues from each of these HVRs are noted below.

Loop	Kabat	AbM	Chothia	Contact
L1	L24-L34	L24-L34	L26-L32	L30-L36
L2	L50-L56	L50-L56	L50-L52	L46-L55
L3	L89-L97	L89-L97	L91-L96	L89-L96
H1	H31-H35B	H26-H35B	H26-H32	H30-H35B
	(Kabat Numbering)			
H1	H31-H35	H26-H35	H26-H32	H30-H35
	(Chothia Numbering)			
H2	H50-H65	H50-H58	H53-H55	H47-H58
H3	H95-H102	H95-H102	H96-H101	H93-H101

[0098] HVRs may comprise "extended HVRs" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102, or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat et al., supra, for each of these definitions.

[0099] "Framework" or "FR" residues are those variable domain residues other than the HVR residues as herein defined.

[0100] The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., *supra*. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

[0101] The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (e.g, Kabat *et al.*, *Sequences of Immunological Interest*. 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The "EU numbering system" or "EU index" is generally used when referring to a residue in an immunoglobulin heavy chain

constant region (e.g., the EU index reported in Kabat *et al., supra*). The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

[0102] The expression "linear antibodies" refers to the antibodies described in Zapata et al. (1995 *Protein Eng*, 8(10):1057-1062). Briefly, these antibodies comprise a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

[0103] As used herein, "library" refers to a plurality of antibody or antibody fragment sequences (for example, polypeptides of the invention), or the nucleic acids that encode these sequences, the sequences being different in the combination of variant amino acids that are introduced into these sequences according to the methods of the invention.

[0104] "Phage display" is a technique by which variant polypeptides are displayed as fusion proteins to at least a portion of coat protein on the surface of phage, e.g., filamentous phage, particles. A utility of phage display lies in the fact that large libraries of randomized protein variants can be rapidly and efficiently sorted for those sequences that bind to a target antigen with high affinity. Display of peptide and protein libraries on phage has been used for screening millions of polypeptides for ones with specific binding properties. Polyvalent phage display methods have been used for displaying small random peptides and small proteins through fusions to either gene III or gene VIII of filamentous phage. Wells and Lowman (1992) *Curr. Opin. Struct. Biol.* 3:355-362, and references cited therein. In a monovalent phage display, a protein or peptide library is fused to a gene III or a portion thereof, and expressed at low levels in the presence of wild type gene III protein so that phage particles display one copy or none of the fusion proteins. Avidity effects are reduced relative to polyvalent phage so that sorting is on the basis of intrinsic ligand affinity, and phagemid vectors are used, which simplify DNA manipulations. Lowman and Wells (1991) *Methods: A companion to Methods in Enzymology* 3:205-0216.

[0105] A "phagemid" is a plasmid vector having a bacterial origin of replication, e.g., Co1E1, and a copy of an intergenic region of a bacteriophage. The phagemid may be used on any known bacteriophage, including filamentous bacteriophage and lambdoid bacteriophage. The plasmid will also generally contain a selectable marker for antibiotic resistance. Segments of DNA cloned into these vectors can be propagated as plasmids. When cells harboring these vectors are provided with all genes necessary for the production of phage particles, the mode of replication of the plasmid changes to rolling circle replication to generate copies of one strand of the plasmid DNA and package phage particles. The phagemid may form infectious or non-infectious phage particles. This term includes phagemids which contain a phage coat protein

gene or fragment thereof linked to a heterologous polypeptide gene as a gene fusion such that the heterologous polypeptide is displayed on the surface of the phage particle.

II. Modes for Carrying Out the Invention

[0106] The invention herein relates to a stable aqueous formulation comprising an antibody. The antibody in the formulation is prepared using techniques available in the art for generating antibodies, exemplary methods of which are described in more detail in the following sections.

[0107] The antibody is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens are also contemplated.

[0108] Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include molecules such as vascular endothelial growth factor (VEGF); ox-LDL; ox-ApoB100; renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tssue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tssue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF- β 1, TGF- β 2, TGF- β 3, TGF-β 4, or TGF- β 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP);

an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrns such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

[0109] In certain embodiments of the invention, the molecular targets for antibodies encompassed by the invention include VEGF. In one embodiment, the antibody herein is one which binds to human VEGF.

A. Preparation of the Formulation

[0110] After preparation of the antibody of interest (e.g., techniques for producing antibodies which can be formulated as disclosed herein will be elaborated below and are known in the art), the pharmaceutical formulation comprising it is prepared. In certain embodiments, the antibody to be formulated has not been subjected to prior lyophilization and the formulation of interest herein is an aqueous formulation. In certain embodiments, the antibody is a full length antibody. In one embodiment, the antibody in the formulation is an antibody fragment, such as an $F(ab')_2$, in which case problems that may not occur for the full length antibody (such as clipping of the antibody to Fab) may need to be addressed. The therapeutically effective amount of antibody present in the formulation is determined by taking into account the desired dose volumes and mode(s) of administration, for example. From about 0.1 mg/mL to about 250 mg/mL, or from about 10 mg/mL to about 200 mg/mL or from about 50 mg/mL to about 175 mg/mL is an exemplary antibody concentration in the formulation.

[0111] An aqueous formulation is prepared comprising the antibody in a pH-buffered solution. The buffer of this invention has a pH in the range from about 4.0 to about 6.5. In certain embodiments the pH is in the range from pH 4.25 to 6.25, or in the range from pH 4.5 to 6.0, or in the range from pH 4.75 to 5.75, or in the range from pH 5.0 to 5.5, or in the range from pH 5.1 to 5.4. In certain embodiments of the invention, the formulation has a pH of 5.2 or about 5.2. Examples of buffers that will control the pH within this range include acetate (e.g. arginine acetate or sodium acetate), succinate (such as arginine succinate or sodium succinate), gluconate, citrate and other organic acid buffers and combinations thereof. The buffer concentration can be from about 1 mM to about 600 mM, depending, for example, on the buffer and the desired isotonicity of the formulation. In certain embodiments, the buffer contains arginine in the concentration of 50 mM to 500 mM, 75 mM to 400 mM, 100 mM to 250 mM, 120 mM to 240 mM, 150 mM to 225 mM, or 175 mM to 210 mM. In certain

embodiments of the invention, the buffer contains arginine in the concentration of 200 mM or about 200 mM. In one embodiment, the buffer is arginine acetate (e.g., at 200 mM or about 200 mM), pH 5.2.

[0112] A surfactant can optionally be added to the antibody formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (e.g. polysorbates 20, 80 etc) or poloxamers (e.g. poloxamer 188). The amount of surfactant added is such that it reduces aggregation of the formulated antibody and/or minimizes the formation of particulates in the formulation and/or reduces adsorption. For example, the surfactant may be present in the formulation in an amount from about 0.001% to about 0.5%, from about 0.005% to about 0.2% , from about 0.01% to about 0.1%, or from about 0.02% to about 0.06%, or about 0.03% to about 0.05%. In certain embodiments, the surfactant is present in the formuation in an amount of 0.04% or about 0.04%. In one embodiment, the formulation does not comprise a surfactant.

[0113] In one embodiment, the formulation contains the above-identified agents (e.g., antibody, buffer, and/or surfactant) and is essentially free of one or more preservatives, such as benzyl alcohol, phenol, m-cresol, chlorobutanol and benzethonium Cl. In another embodiment, a preservative may be included in the formulation, particularly where the formulation is a multidose formulation. The concentration of preservative may be in the range from about 0.1% to about 2%, preferably from about 0.5% to about 1%. One or more other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) may be included in the formulation provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; co-solvents; anti-oxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g. Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions. Exemplary pharmaceutically acceptable carriers herein further include insterstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rHuPH20 (HYLENEX®, Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rHuPH20, are described in US Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

[0114] While the various descriptions of chelators herein often focus on EDTA, it will be appreciated that other metal ion chelators are also encompassed within the invention. Metal

ion chelators are well known by those of skill in the art and include, but are not necessarily limited to aminopolycarboxylates, EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid), NTA (nitrilotriacetic acid), EDDS (ethylene diamine disuccinate), PDTA (1,3-propylenediaminetetraacetic acid), DTPA (diethylenetriaminepentaacetic acid), ADA (beta-alaninediacetic acid), MGCA (methylglycinediacetic acid), etc. Additionally, some embodiments herein comprise phosphonates/phosphonic acid chelators.

[0115] The formulation herein may also contain more than one protein as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect the other protein. For example, where the antibody is anti-VEGF, it may be combined with another agent (e.g., a chemotherapeutic agent, and anti-neoplastic agent, and anti.

[0116] The formulations to be used for in vivo administration should be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, preparation of the formulation.

B. Administration of the Formulation

[0117] The formulation is administered to a mammal in need of treatment with the antibody, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. In one embodiment, the formulation is administered to the mammal by intravenous administration. For such purposes, the formulation may be injected using a syringe or via an IV line, for example. In one embodiment, the formulation is administered to the mammal by subcutaneous administration.

[0118] The appropriate dosage ("therapeutcally effective amount") of the antibody will depend, for example, on the condition to be treated, the severity and course of the condition, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, the type of antibody used, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments and may be administered to the patient at any time from diagnosis onwards. The antibody may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

[0119] As a general proposition, the therapeutically effective amount of the antibody administered will be in the range of about 0.1 to about 50 mg/kg of patent body weight whether by one or more administrations, with the typical range of antibody used being about 0.3 to

about 20 mg/kg, preferably about 0.3 to about 15 mg/kg, administered daily, for example. However, other dosage regimens may be useful. In one embodiment, the antagonist is an anti-VEGF antibody that is administered at a dose of about 100 or 400 mg every 1, 2, 3, or 4 weeks or is administered a dose of about 1, 3, 5, 7.5, 10, 15, or 20 mg/kg every 1, 2, 3, or 4 weeks. The dose may be administered as a single dose or as multiple doses (e.g., 2 or 3 doses), such as infusions. The progress of this therapy is easily monitored by conventional techniques.

C. Antibody Preparation

(i) Antigen Preparation

[0120] Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule. Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

(ii) Certain Antibody-Based Methods

[0121] Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

[0122] Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as

protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

[0123] Monoclonal antibodies of the invention can be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), and further described, e.g., in Hongo *et al.*, *Hybridoma*, 14 (3): 253-260 (1995), Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981), and Ni, *Xiandai Mianyixue*, 26(4):265-268 (2006) regarding human-human hybridomas. Additional methods include those described, for example, in U.S. Pat. No. 7,189,826 regarding production of monoclonal human natural IgM antibodies from hybridoma cell lines. Human hybridoma technology (Trioma technology) is described in Vollmers and Brandlein, *Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and Findings in Experimental and Clinical Pharmacology*, 27(3):185-91 (2005).

[0124] For various other hybridoma techniques, see, *e.g.*, US 2006/258841; US 2006/183887 (fully human antibodies), US 2006/059575; US 2005/287149; US 2005/100546; US 2005/026229; and U.S. Pat. Nos. 7,078,492 and 7,153,507. An exemplary protocol for producing monoclonal antibodies using the hybridoma method is described as follows. In one embodiment, a mouse or other appropriate host animal, such as a hamster, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Antibodies are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of a polypeptide of the invention or a fragment thereof, and an adjuvant, such as monophosphoryl lipid A (MPL)/trehalose dicrynomycolate (TDM) (Ribi Immunochem. Research, Inc., Hamilton, MT). A polypeptide of the invention (e.g., antigen) or a fragment thereof may be prepared using methods well known in the art, such as recombinant methods, some of which are further described herein. Serum from immunized animals is assayed for anti-antigen antibodies, and booster immunizations are optionally administered. Lymphocytes from animals producing anti-antigen antibodies are isolated. Alternatively, lymphocytes may be immunized *in vitro*.

[0125] Lymphocytes are then fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. See, *e.g.*, Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986). Myeloma cells may be used that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Exemplary myeloma cells include, but are not limited to, murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution

Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

[0126] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium, e.g., a medium that contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells. Preferably, serum-free hybridoma cell culture methods are used to reduce use of animal-derived serum such as fetal bovine serum, as described, for example, in Even *et al.*, *Trends in Biotechnology*, 24(3), 105-108 (2006).

[0127] Oligopeptides as tools for improving productivity of hybridoma cell cultures are described in Franek, *Trends in Monoclonal Antibody Research*, 111-122 (2005). Specifically, standard culture media are enriched with certain amino acids (alanine, serine, asparagine, proline), or with protein hydrolyzate fractions, and apoptosis may be significantly suppressed by synthetic oligopeptides, constituted of three to six amino acid residues. The peptides are present at millimolar or higher concentrations.

[0128] Culture medium in which hybridoma cells are growing may be assayed for production of monoclonal antibodies that bind to an antibody of the invention. The binding specificity of monoclonal antibodies produced by hybridoma cells may be determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoadsorbent assay (ELISA). The binding affinity of the monoclonal antibody can be determined, for example, by Scatchard analysis. See, *e.g.*, Munson *et al., Anal. Biochem.*, 107:220 (1980).

[0129] After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods. See, *e.g.*, Goding, *supra*. Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, hybridoma cells may be grown *in vivo* as ascites tumors in an animal. Monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein

A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. One procedure for isolation of proteins from hybridoma cells is described in US 2005/176122 and U.S. Pat. No. 6,919,436. The method includes using minimal salts, such as lyotropic salts, in the binding process and preferably also using small amounts of organic solvents in the elution process.

(iii) Certain Library Screening Methods

[0130] Antibodies of the invention can be made by using combinatorial libraries to screen for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are described generally in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001). For example, one method of generating antibodies of interest is through the use of a phage antibody library as described in Lee et al., J. Mol. Biol. (2004), 340(5):1073-93.

[0131] In principle, synthetic antibody clones are selected by screening phage libraries containing phage that display various fragments of antibody variable region (Fv) fused to phage coat protein. Such phage libraries are panned by affinity chromatography against the desired antigen. Clones expressing Fv fragments capable of binding to the desired antigen are adsorbed to the antigen and thus separated from the non-binding clones in the library. The binding clones are then eluted from the antigen, and can be further enriched by additional cycles of antigen adsorption/elution. Any of the antibodies of the invention can be obtained by designing a suitable antigen screening procedure to select for the phage clone of interest followed by construction of a full length antibody clone using the Fv sequences from the phage clone of interest and suitable constant region (Fc) sequences described in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3.

[0132] In certain embodiments, the antigen-binding domain of an antibody is formed from two variable (V) regions of about 110 amino acids, one each from the light (VL) and heavy (VH) chains, that both present three hypervariable loops (HVRs) or complementarity-determining regions (CDRs). Variable domains can be displayed functionally on phage, either as single-chain Fv (scFv) fragments, in which VH and VL are covalently linked through a short, flexible peptide, or as Fab fragments, in which they are each fused to a constant domain and interact non-covalently, as described in Winter *et al.*, *Ann. Rev. Immunol.*, 12: 433-455 (1994). As used herein, scFv encoding phage clones and Fab encoding phage clones are collectively referred to as "Fv phage clones" or "Fv clones."

[0133] Repertoires of VH and VL genes can be separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be searched for antigen-binding clones as described in Winter *et al.*, *Ann. Rev. Immunol.*, 12: 433-455 (1994). Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned to provide a single source of human antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths *et al.*, *EMBO J,* 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning the unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement in vitro as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992).

[0134] In certain embodiments, filamentous phage is used to display antibody fragments by fusion to the minor coat protein pIII. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains are connected on the same polypeptide chain by a flexible polypeptide spacer, e.g. as described by Marks *et al.*, *J. Mol. Biol.,* 222: 581-597 (1991), or as Fab fragments, in which one chain is fused to pIII and the other is secreted into the bacterial host cell periplasm where assembly of a Fab-coat protein structure which becomes displayed on the phage surface by displacing some of the wild type coat proteins, e.g. as described in Hoogenboom *et al.*, *Nucl. Acids Res.*, 19: 4133-4137 (1991).

[0135] In general, nucleic acids encoding antibody gene fragments are obtained from immune cells harvested from humans or animals. If a library biased in favor of anti-antigen clones is desired, the subject is immunized with antigen to generate an antibody response, and spleen cells and/or circulating B cells other peripheral blood lymphocytes (PBLs) are recovered for library construction. In one embodiment, a human antibody gene fragment library biased in favor of anti-antigen clones is obtained by generating an anti-antigen antibody response in transgenic mice carrying a functional human immunoglobulin gene array (and lacking a functional endogenous antibody production system) such that antigen immunization gives rise to B cells producing human antibodies against antigen. The generation of human antibody-producing transgenic mice is described below.

[0136] Additional enrichment for anti-antigen reactive cell populations can be obtained by using a suitable screening procedure to isolate B cells expressing antigen-specific membrane bound antibody, e.g., by cell separation using antigen affinity chromatography or adsorption of cells to fluorochrome-labeled antigen followed by flow-activated cell sorting (FACS).

[0137] Alternatively, the use of spleen cells and/or B cells or other PBLs from an unimmunized donor provides a better representation of the possible antibody repertoire, and also permits the construction of an antibody library using any animal (human or non-human) species in which antigen is not antigenic. For libraries incorporating in vitro antibody gene construction, stem cells are harvested from the subject to provide nucleic acids encoding unrearranged antibody gene segments. The immune cells of interest can be obtained from a variety of animal species, such as human, mouse, rat, lagomorpha, luprine, canine, feline, porcine, bovine, equine, and avian species, etc.

[0138] Nucleic acid encoding antibody variable gene segments (including VH and VL segments) are recovered from the cells of interest and amplified. In the case of rearranged VH and VL gene libraries, the desired DNA can be obtained by isolating genomic DNA or mRNA from lymphocytes followed by polymerase chain reaction (PCR) with primers matching the 5' and 3' ends of rearranged VH and VL genes as described in Orlandi *et al.*, *Proc. Natl. Acad. Sci. (USA),* 86: 3833-3837 (1989), thereby making diverse V gene repertoires for expression. The V genes can be amplified from cDNA and genomic DNA, with back primers at the 5' end of the exon encoding the mature V-domain and forward primers based within the J-segment as described in Orlandi *et al.* (1989) and in Ward *et al., Nature*, 341: 544-546 (1989). However, for amplifying from cDNA, back primers can also be based in the leader exon as described in Jones *et al.*, *Biotechnol.*, 9: 88-89 (1991), and forward primers within the constant region as described in Sastry *et al.*, *Proc. Natl. Acad. Sci. (USA),* 86: 5728-5732 (1989). To maximize complementarity, degeneracy can be incorporated in the primers as described in Orlandi *et al.* (1989) or Sastry *et al.* (1989). In certain embodiments, library diversity is maximized by using PCR primers targeted to each V-gene family in order to amplify all available VH and VL arrangements present in the immune cell nucleic acid sample, e.g. as described in the method of Marks *et al.*, *J. Mol. Biol.*, 222: 581-597 (1991) or as described in the method of Orum *et al.*, *Nucleic Acids Res.*, 21: 4491-4498 (1993). For cloning of the amplified DNA into expression vectors, rare restriction sites can be introduced within the PCR primer as a tag at one end as described in Orlandi *et al.* (1989), or by further PCR amplification with a tagged primer as described in Clackson *et al.*, *Nature*, 352: 624-628 (1991).

[0139] Repertoires of synthetically rearranged V genes can be derived in vitro from V gene segments. Most of the human VH-gene segments have been cloned and sequenced (reported in Tomlinson *et al.*, *J. Mol. Biol.,* 227: 776-798 (1992)), and mapped (reported in Matsuda *et al.*, *Nature Genet.,* **3**: 88-94 (1993); these cloned segments (including all the major conformations of the H1 and H2 loop) can be used to generate diverse VH gene repertoires with PCR primers encoding H3 loops of diverse sequence and length as described in

Hoogenboom and Winter, *J. Mol. Biol.,* 227: 381-388 (1992). VH repertoires can also be made with all the sequence diversity focused in a long H3 loop of a single length as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, 89: 4457-4461 (1992). Human Vκ and Vλ segments have been cloned and sequenced (reported in Williams and Winter, *Eur. J. Immunol.*, 23: 1456-1461 (1993)) and can be used to make synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, will encode antibodies of considerable structural diversity. Following amplification of V-gene encoding DNAs, germline V-gene segments can be rearranged in vitro according to the methods of Hoogenboom and Winter, *J. Mol. Biol.,* 227: 381-388 (1992).

[0140] Repertoires of antibody fragments can be constructed by combining VH and VL gene repertoires together in several ways. Each repertoire can be created in different vectors, and the vectors recombined in vitro, e.g., as described in Hogrefe *et al.*, *Gene*, 128: 119-126 (1993), or in vivo by combinatorial infection, e.g., the loxP system described in Waterhouse *et al.*, *Nucl. Acids Res.,* 21: 2265-2266 (1993). The *in vivo* recombination approach exploits the two-chain nature of Fab fragments to overcome the limit on library size imposed by *E. coli* transformation efficiency. Naive VH and VL repertoires are cloned separately, one into a phagemid and the other into a phage vector. The two libraries are then combined by phage infection of phagemid-containing bacteria so that each cell contains a different combination and the library size is limited only by the number of cells present (about 10^{12} clones). Both vectors contain in vivo recombination signals so that the VH and VL genes are recombined onto a single replicon and are co-packaged into phage virions. These huge libraries provide large numbers of diverse antibodies of good affinity (K_d^{-1} of about 10^{-8} M).

[0141] Alternatively, the repertoires may be cloned sequentially into the same vector, e.g. as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA,* 88: 7978-7982 (1991), or assembled together by PCR and then cloned, e.g. as described in Clackson *et al.*, *Nature*, 352: 624-628 (1991). PCR assembly can also be used to join VH and VL DNAs with DNA encoding a flexible peptide spacer to form single chain Fv (scFv) repertoires. In yet another technique, "in cell PCR assembly" is used to combine VH and VL genes within lymphocytes by PCR and then clone repertoires of linked genes as described in Embleton *et al.*, *Nucl. Acids Res.*, 20: 3831-3837 (1992).

[0142] The antibodies produced by naive libraries (either natural or synthetic) can be of moderate affinity (K_d^{-1} of about 10^6 to 10^7 M^{-1}), but affinity maturation can also be mimicked *in vitro* by constructing and reselecting from secondary libraries as described in Winter *et al.* (1994), *supra*. For example, mutation can be introduced at random in vitro by using error-prone polymerase (reported in Leung *et al.*, *Technique,* **1:** 11-15 (1989)) in the method of

Hawkins *et al.*, *J. Mol. Biol.,* 226: 889-896 (1992) or in the method of Gram *et al.*, *Proc. Natl. Acad. Sci USA*, 89: 3576-3580 (1992). Additionally, affinity maturation can be performed by randomly mutating one or more CDRs, e.g. using PCR with primers carrying random sequence spanning the CDR of interest, in selected individual Fv clones and screening for higher affinity clones. WO 9607754 (published 14 March 1996) described a method for inducing mutagenesis in a complementarity determining region of an immunoglobulin light chain to create a library of light chain genes. Another effective approach is to recombine the VH or VL domains selected by phage display with repertoires of naturally occurring V domain variants obtained from unimmunized donors and screen for higher affinity in several rounds of chain reshuffling as described in Marks *et al.*, *Biotechnol.,* 10: 779-783 (1992). This technique allows the production of antibodies and antibody fragments with affinities of about 10^{-9} M or less.

[0143] Screening of the libraries can be accomplished by various techniques known in the art. For example, antigen can be used to coat the wells of adsorption plates, expressed on host cells affixed to adsorption plates or used in cell sorting, or conjugated to biotin for capture with streptavidin-coated beads, or used in any other method for panning phage display libraries.

[0144] The phage library samples are contacted with immobilized antigen under conditions suitable for binding at least a portion of the phage particles with the adsorbent. Normally, the conditions, including pH, ionic strength, temperature and the like are selected to mimic physiological conditions. The phages bound to the solid phase are washed and then eluted by acid, e.g. as described in Barbas *et al.*, *Proc. Natl. Acad. Sci USA*, 88: 7978-7982 (1991), or by alkali, e.g. as described in Marks *et al.*, *J. Mol. Biol.,* 222: 581-597 (1991), or by antigen competition, e.g. in a procedure similar to the antigen competition method of Clackson *et al.*, *Nature*, 352: 624-628 (1991). Phages can be enriched 20-1,000-fold in a single round of selection. Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection.

[0145] The efficiency of selection depends on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with antigen. Antibodies with fast dissociation kinetics (and weak binding affinities) can be retained by use of short washes, multivalent phage display and high coating density of antigen in solid phase. The high density not only stabilizes the phage through multivalent interactions, but favors rebinding of phage that has dissociated. The selection of antibodies with slow dissociation kinetics (and good binding affinities) can be promoted by use of long washes and monovalent phage display as described in Bass *et al.*,

Proteins, 8: 309-314 (1990) and in WO 92/09690, and a low coating density of antigen as described in Marks *et al.*, *Biotechnol.,* 10: 779-783 (1992).

[0146] It is possible to select between phage antibodies of different affinities, even with affinities that differ slightly, for antigen. However, random mutation of a selected antibody (e.g. as performed in some affinity maturation techniques) is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting antigen, rare high affinity phage could be competed out. To retain all higher affinity mutants, phages can be incubated with excess biotinylated antigen, but with the biotinylated antigen at a concentration of lower molarity than the target molar affinity constant for antigen. The high affinity-binding phages can then be captured by streptavidin-coated paramagnetic beads. Such "equilibrium capture" allows the antibodies to be selected according to their affinities of binding, with sensitivity that permits isolation of mutant clones with as little as two-fold higher affinity from a great excess of phages with lower affinity. Conditions used in washing phages bound to a solid phase can also be manipulated to discriminate on the basis of dissociation kinetics.

[0147] Anti-antigen clones may be selected based on activity. In certain embodiments, the invention provides anti-antigen antibodies that bind to living cells that naturally express antigen or bind to free floating antigen or antigen attached to other cellular structures. Fv clones corresponding to such anti- antigen antibodies can be selected by (1) isolating anti-antigen clones from a phage library as described above, and optionally amplifying the isolated population of phage clones by growing up the population in a suitable bacterial host; (2) selecting antigen and a second protein against which blocking and non-blocking activity, respectively, is desired; (3) adsorbing the anti- antigen phage clones to immobilized antigen; (4) using an excess of the second protein to elute any undesired clones that recognize antigen - binding determinants which overlap or are shared with the binding determinants of the second protein; and (5) eluting the clones which remain adsorbed following step (4). Optionally, clones with the desired blocking/non-blocking properties can be further enriched by repeating the selection procedures described herein one or more times.

[0148] DNA encoding hybridoma-derived monoclonal antibodies or phage display Fv clones of the invention is readily isolated and sequenced using conventional procedures (e.g. by using oligonucleotide primers designed to specifically amplify the heavy and light chain coding regions of interest from hybridoma or phage DNA template). Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of the desired monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in

bacteria of antibody-encoding DNA include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5: 256 (1993) and Pluckthun, *Immunol. Revs*, 130: 151 (1992).

[0149] DNA encoding the Fv clones of the invention can be combined with known DNA sequences encoding heavy chain and/or light chain constant regions (e.g. the appropriate DNA sequences can be obtained from Kabat *et al.*, supra) to form clones encoding full or partial length heavy and/or light chains. It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. An Fv clone derived from the variable domain DNA of one animal (such as human) species and then fused to constant region DNA of another animal species to form coding sequence(s) for "hybrid," full length heavy chain and/or light chain is included in the definition of "chimeric" and "hybrid" antibody as used herein. In certain embodiments, an Fv clone derived from human variable DNA is fused to human constant region DNA to form coding sequence(s) for full- or partial-length human heavy and/or light chains.

[0150] DNA encoding anti-antigen antibody derived from a hybridoma of the invention can also be modified, or example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of homologous murine sequences derived from the hybridoma clone (e.g. as in the method of Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81: 6851-6855 (1984)). DNA encoding a hybridoma- or Fv clone-derived antibody or fragment can be further modified by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In this manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of the Fv clone or hybridoma clone-derived antibodies of the invention.

(iv) Humanized and Human Antibodies

[0151] Various methods for humanizing non-human antibodies are known in the art. For example, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are

typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0152] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad Sci. USA*, 89:4285 (1992); Presta et al., *J. Immnol.*, 151:2623 (1993)).

[0153] It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to one embodiment of the method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

[0154] Human antibodies of the invention can be constructed by combining Fv clone variable domain sequence(s) selected from human-derived phage display libraries with known human constant domain sequence(s) as described above. Alternatively, human monoclonal antibodies of the invention can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner *et al.*, *J. Immunol.*, 147: 86 (1991).

[0155] It is possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. *See, e.g.,* Jakobovits et al, *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992).

[0156] Gene shuffling can also be used to derive human antibodies from non-human, e.g. rodent, antibodies, where the human antibody has similar affinities and specificities to the starting non-human antibody. According to this method, which is also called "epitope imprinting", either the heavy or light chain variable region of a non-human antibody fragment obtained by phage display techniques as described herein is replaced with a repertoire of human V domain genes, creating a population of non-human chain/human chain scFv or Fab chimeras. Selection with antigen results in isolation of a non-human chain/human chain chimeric scFv or Fab wherein the human chain restores the antigen binding site destroyed upon removal of the corresponding non-human chain in the primary phage display clone, i.e. the epitope governs (imprints) the choice of the human chain partner. When the process is repeated in order to replace the remaining non-human chain, a human antibody is obtained (see PCT WO 93/06213 published April 1, 1993). Unlike traditional humanization of non-human antibodies by CDR grafting, this technique provides completely human antibodies, which have no FR or CDR residues of non-human origin.

(v) Antibody Fragments

[0157] Antibody fragments may be generated by traditional means, such as enzymatic digestion, or by recombinant techniques. In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors. For a review of certain antibody fragments, see Hudson et al. (2003) *Nat. Med.* 9:129-134.

[0158] Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan et al., *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody fragments can all be expressed in and secreted from E. coli, thus allowing the facile production of large

amounts of these fragments. Antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Fab and $F(ab')_2$ fragment with increased in vivo half-life comprising salvage receptor binding epitope residues are described in U.S. Pat. No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In certain embodiments, an antibody is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. Nos. 5,571,894; and 5,587,458. Fv and scFv are the only species with intact combining sites that are devoid of constant regions; thus, they may be suitable for reduced nonspecific binding during in vivo use. scFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an scFv. See *Antibody Engineering*, ed. Borrebaeck, supra. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870, for example. Such linear antibodies may be monospecific or bispecific.

(vi) Multispecific Antibodies

[0159] Multspecific antibodies have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two different epitopes (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

[0160] Methods for making bispecific antibodies are known in the art. Traditonal production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., Nature, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

[0161] According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant

domain, comprising at least part of the hinge, CH2, and CH3 regions. It is typical to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

[0162] In one embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies *see,* for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

[0163] According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. One interface comprises at least a part of the C_H 3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

[0164] Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any

convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

[0165] Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

[0166] Recent progress has facilitated the direct recovery of Fab'-SH fragments from E. coli, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from E. coli and subjected to directed chemical coupling in vitro to form the bispecific antibody.

[0167] Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Nati. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. *See* Gruber et al, *J. Immunol*, 152:5368 (1994).

[0168] Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. *Tuft et al. J. Immunol.* 147: 60 (1991).

(vii) Single-Domain Antibodies

[0169] In some embodiments, an antibody of the invention is a single-domain antibody. A single-domain antibody is a single polypetide chain comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; *see, e.g.*, U.S. Patent No. 6,248,516 B1). In one embodiment, a single-domain antibody consists of all or a portion of the heavy chain variable domain of an antibody.

(viii) Antibody Variants

[0170] In some embodiments, amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody may be prepared by introducing appropriate changes into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid alterations may be introduced in the subject antibody amino acid sequence at the time that sequence is made.

(ix) Antibody Derivatives

[0171] The antibodies of the invention can be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. In certain embodiments, the moieties suitable for derivatization of the antibody are water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number

and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

(x) Vectors, Host Cells, and Recombinant Methods

[0172] Antibodies may also be produced using recombinant methods. For recombinant production of an anti-antigent antibody, nucleic acid encoding the antibody is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody may be readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(a) Signal sequence component

[0173] An antibody of the invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (*e.g.,* cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process a native antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.,* the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

(b) Origin of replication

[0174] Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such

sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

(c) Selection gene component

[0175] Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.,* ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.,* the gene encoding D-alanine racemase for *Bacilli.*

[0176] One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

[0177] Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up antibody-encoding nucleic acid, such as DHFR, glutamine synthetase (GS), thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

[0178] For example, cells transformed with the DHFR gene are identified by culturing the transformants in a culture medium containing methotrexate (Mtx), a competitive antagonist of DHFR. Under these conditions, the DHFR gene is amplified along with any other co-transformed nucleic acid. A Chinese hamster ovary (CHO) cell line deficient in endogenous DHFR activity (e.g., ATCC CRL-9096) may be used.

[0179] Alternatively, cells transformed with the GS gene are identified by culturing the transformants in a culture medium containing L-methionine sulfoximine (Msx), an inhibitor of GS. Under these conditions, the GS gene is amplified along with any other co-transformed nucleic acid. The GS selection/amplification system may be used in combination with the DHFR selection/amplification system described above.

[0180] Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding an antibody of interest, wild-type DHFR gene, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection

agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.,* kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

[0181] A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al., Nature*, 282:39 (1979)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

[0182] In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al., Bio/Technology*, 9:968-975 (1991).

(d) Promoter component

[0183] Expression and cloning vectors generally contain a promoter that is recognized by the host organism and is operably linked to nucleic acid encoding an antibody. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter , β-lactamase and lactose promoter systems, alkaline phosphatase promoter, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding an antibody.

[0184] Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

[0185] Examples of suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phospho-

fructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

[0186] Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

[0187] Antibody transcription from vectors in mammalian host cells can be controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus, Simian Virus 40 (SV40), or from heterologous mammalian promoters, *e.g.,* the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

[0188] The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

(e) Enhancer element component

[0189] Transcription of a DNA encoding an antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into

the vector at a position 5' or 3' to the antibody-encoding sequence, but is preferably located at a site 5' from the promoter.

(f) Transcription termination component

[0190] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

(g) Selection and transformation of host cells

[0191] Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

[0192] Full length antibody, antibody fusion proteins, and antibody fragments can be produced in bacteria, in particular when glycosylation and Fc effector function are not needed, such as when the therapeutic antibody is conjugated to a cytotoxic agent (e.g., a toxin) that by itself shows effectiveness in tumor cell destruction. Full length antibodies have greater half life in circulation. Production in E. coli is faster and more cost efficient. For expression of antibody fragments and polypeptides in bacteria, see, e.g., U.S. 5,648,237 (Carter et. al.), U.S. 5,789,199 (Joly et al.), U.S. 5,840,523 (Simmons et al.), which describes translation initiation region (TIR) and signal sequences for optimizing expression and secretion. *See also* Charlton, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in *E. coli*. After expression, the antibody may be isolated from the E. coli cell paste in a soluble fraction and can be purified

through, e.g., a protein A or G column depending on the isotype. Final purification can be carried out similar to the process for purifying antibody expressed e.g,, in CHO cells.

[0193] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K . thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g., Neurospora, Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*. For a review discussing the use of yeasts and filamentous fungi for the production of therapeutic proteins, see, e.g., Gerngross, *Nat. Biotech.* 22:1409-1414 (2004).

[0194] Certain fungi and yeast strains may be selected in which glycosylation pathways have been "humanized," resulting in the production of an antibody with a partially or fully human glycosylation pattern. *See, e.g.*, Li et al., *Nat. Biotech.* 24:210-215 (2006) (describing humanization of the glycosylation pathway in *Pichia pastoris*); and Gerngross et al., *supra*.

[0195] Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the invention, particularly for transfection of *Spodoptera frugiperda* cells.

[0196] Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, duckweed (*Lemnaceae*), alfalfa (*M. truncatula*), and tobacco can also be utilized as hosts. *See, e.g.*, US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants).

[0197] Vertebrate cells may be used as hosts, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651);

human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)) ; baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2). Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including $DHFR^-$ CHO cells (Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); and myeloma cell lines such as NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki and Wu, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 255-268.

[0198] Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

(h) Culturing the host cells

[0199] The host cells used to produce an antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art.

The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(xi) Purification of antibody

[0200] When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0201] The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, hydrophobic interaction chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being among one of the typically preferred purification steps. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

[0202] In general, various methodologies for preparing antibodies for use in research, testing, and clinicalare well-established in the art, consistent with the above-described methodologies and/or as deemed appropriate by one skilled in the art for a particular antibody of interest.

D. Selecting Biologically Active Antibodies

[0203] Antibodies produced as described above may be subjected to one or more "biological activity" assays to select an antibody with beneficial properties from a therapeutic perspective. The antibody may be screened for its ability to bind the antigen against which it was raised. For example, for an anti-VEGF antibody, as shown in the example below, the antigen binding properties of the antibody can be evaluated in an assay that detects the ability to bind to VEGF.

[0204] In another embodiment, the affinity of the antibody may be determined by saturation binding; ELISA; and/or competition assays (e.g. RIA's), for example.

[0205] Also, the antibody may be subjected to other biological activity assays, e.g., in order to evaluate its effectiveness as a therapeutic. Such assays are known in the art and depend on the target antigen and intended use for the antibody.

[0206] To screen for antibodies which bind to a particular epitope on the antigen of interest (e.g., those which block binding of the anti-VEGF antibody of the example to VEGF), a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping, e.g. as described in Champe et al., *J. Biol. Chem.* 270:1388-1394 (1995), can be performed to determine whether the antibody binds an epitope of interest.

E. Articles of Manufacture

[0207] In another embodiment of the invention, an article of manufacture is provided comprising a container which holds the aqueous pharmaceutical formulation of the invention and optionally provides instructions for its use. Suitable containers include, for example, bottles, vials and syringes. The container may be formed from a variety of materials such as glass or plastic. An exemplary container is a 3-20 cc single use glass vial. Alternatively, for a multidose formulation, the container may be 3-100 cc glass vial. The container holds the formulation and the label on, or associated with, the container may indicate directions for use. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

[0208] The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature and patent citations are incorporated herein by reference.

[0209] The specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

[0210] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Stable anti-VEGF antibody Liquid Formulations

[0211] This examples describe the development and stability testing of stable liquid formulations comprising anti-VEGF antibody at protein concentrations in the range from about 20 mg/mL-200 mg/mL in various liquid formulations comprising comprising histidine, arginine, acetate, or sodium chloride. One milliliter of each formulation in a 3cc glass vials was stored at 40^0C and the stability was assessed at 1, 2, and 4 weeks. The stability of anti-VEGF was monitored by several assays including UV (for concentration and turbidity), size exclusion chromatography (SEC) for size variant analysis, imaged capillary isoelectric focusing (icIEF) for charge variant analysis, CE-SDS for size distribution and binding assay for activity. After four weeks of stability testing, our results indicate that anti-VEGF is stable in 200 mM Arginine Acetate, 150 mM Sodium Chloride, 0.04% PS20, pH 5.2.

[0212] The stability (e.g., aggregate formation, viscosity, etc.) of anti-VEGF was investigated in various liquid formulations comprising histidine, sodium chloride, arginine, and acetate. The stability of anti-VEGF was monitored by several assays including, size exclusion chromatography (SEC) for aggregate formation analysis. Our results indicate that anti-VEGF is stable at about pH 5.2 in arginine-containing buffers.

[0213] Anti-VEGF was formulated into different buffers by dialysis using Slide-a-Lyzer® cassettes to achieve the final concentrations listed in Table 1. Each formulation was sterile filtered with 0.22μm Steriflip® filter units and aseptically filled into autoclaved vials,

stoppered, and sealed. Samples were placed at 2-8°C, 25°C, and 40°C and stability studies were conducted at select temperatures.

Table 1: Formulations

Formulation	
A	51 mM sodium phosphate, 159 mM Trehalose, 0.04% PS20, pH 6.2
B	200 mM Arginine Acetate, 0.04% PS20, pH 5.2
C	20 mM Sodium Acetate, 240 mM Sucrose, 0.04% PS20, pH 5.2
D	20 mM Histidine Chloride, 200 mM Arginine Chloride, 0.04% PS20, pH 5.2

METHODS

[0214] *pH:* A 200 µL volume of each sample was placed in a 1.5ml Eppendorf tubes at an ambient temperature and their pH was measured using Thermo Orion pH meter equipped with a Ross® semi-micro electrode. The pH meter was calibrated using Thermo Orion buffer standards pH 4.0, 5.0 and 7.0.

[0215] *Viscosity:* Shear viscosity was measured using an Anton Paar Physica MCR300 rheometer with a 25 mm cone (CP 25-1) set at a height of 0.049 mm. 75 µL of each sample was loaded onto a Peltier plate at 25°C and measured 10 times per 100s interval at a constant shear rate of 1000 1/s.

[0216] *Size Exclusion Exchange Chromatography (SEC)*: Size exclusion chromatography was performed to quantitate total aggregate levels (with neat injections) and slow-dissociating aggregate levels (with dilute injections). Dilute injections were diluted with mobile phase buffer (0.20M potassium phosphate, 0.25M potassium chloride, pH 6.2) to 0.5 mg/mL. All samples were incubated at 30°C for 24 hours prior to analysis. 10µL of each neat sample and 100µL of each dilute sample were injected onto a TSK G3000SWXL, 7.8 X 300mm column (TOSOHAAS, part no. 08541) using an Agilent 1100 HPLC system. The autosampler was kept at 30°C while the column was kept at ambient temperature. Flow rate was 0.5 mL/min and total run time per sample was 30 minutes. Data was analyzed with sample absorbance at 280 nm using HP Chemstation.

[0217] *Ion Exchange Chromatography (IEC):* Ion exchange chromatography was performed to quantitate charged variants in carboxypeptidase B (CpB)-digested samples. Samples were diluted to 1 mg/mL with Solvent A (20 mM N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) buffer, pH 6.5), treated with a 1% w/w addition of 1 mg/mL CpB, and incubated for 20 minutes at 37°C. 50µL of each sample was then injected onto a

Dionex ProPac WCX-10, 4.6 X 250 mm column using an Agilent 1100 HPLC system. Autosampler temperature was kept at 2-8°C while the column was kept at 40°C. Flow rate was 0.5 mL/min while using a gradient of Solvent A and Solvent B (200 mM sodium chloride in Solvent A) over 90 minutes, as listed in the test procedure. Data was analyzed with sample absorbance at 280 nm using HP Chemstation.

[0218] *Turbidity Assay*: To monitor turbidity the optical density of each formulation was measured at 350 nm using an Agilent 8453 UV-VIS spectrophotmeter. All samples were analyzed without dilution using a 1 cm pathlength quartz cuvette.

[0219] *-20°C and Freeze Thaw Stability Studies*: Formulations A-D are aseptically filled into 316 L stainless steel mini-cans (15 mL/minican). All samples are stored at -20°C for varying amounts of time (e.g., 24, 48, 72, or more hours; 4, 5, 6, 7, or more days; 2, 3, 4, 5, 6, 7, 8, or more weeks; 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or more months, and continuously sampled aseptically under a laminar flow hood. In addition, formulations A-D are filled into 6 cc glass vials and stored at -20°C. Each vial is subjected to five freeze thaw cycles and analyzed using SEC, IEC and turbidity assays. The freeze-thaw cycle entails storage for at least 24 hours at -20°C followed by storage for at least 24 hours at 5°C.

RESULTS AND DISCUSSION

[0220] This study investigated the stability (e.g., aggregate formation, viscosity, chemical stability etc.) of different concentrations of anti-VEGF in an arginine-based formulation. SEC and IEC were used to monitor stability of anti-VEGF at stressed and accelerated storage conditions. Aggregation and viscosity of the anti-VEGF was measured and set forth in Table 2 below and illustrated in Figures 2 and 4.

Table 2: physical properties of formulations studied

Formulation	[Protein] (mg/ml)	%Total Aggregate	Viscosity (cP)
A	25	7	
A	30	8	
A	100	10	
A	150	13	
B	20	N/D	1.3
B	50	2.6	1.9
B	100	3.8	4.2
B	110	N/D	4.7

B	125	N/D	6.1
B	150	N/D	9.5
C	20	N/D	1.6
C	50	3	2.3
C	100	5.3	5.7
C	125	N/D	12.4
C	150	N/D	23.5
C	175	N/D	52
D	20	N/D	1.3
D	50	2.1	1.8
D	100	3.4	4.3
D	125	N/D	6
D	150	N/D	11.3
D	175	N/D	17.7

[0221] *Aggregation of all Formulations at 40°C:* The amount of total aggregate and dimer formed in each anti-VEGF formulation after storage for 0, 1, 2, and 4 weeks at 40°C was measured and is set forth in Tables 3, 4, and 5 below and illustrated in Figures 1 and 3.

Table 3: Total aggregate after storage at 40^0C

Sample	%Total Aggregate 4 weeks
A	10.3
B	5
C	7.1
D	10.8

Table 4: Total aggregate after storage at 40^0C

Sample	% Total Aggregate 0 weeks	% Total Aggregate 1 week	% Total Aggregate 2 weeks	% Total Aggregate 4 weeks
B	3.9	4.7	5.3	6.1
C	5.4	6.8	8.3	8.1
D	3.4	5.2	8.4	13.1

Table 5: Dimer after storage at 40^{0}C

Sample	% Dimer 0 weeks	% Dimer 1 week	% Dimer 2 weeks	% Dimer 4 weeks
B	3.3	3.9	4.4	5.0
C	4.7	6.0	7.4	7.1
D	2.8	4.2	6.7	10.8

Aggregation of Formulations at 25°C: The amount of total aggregate and dimer formed in each anti-VEGF formulation (100 mg/ml) after storage for 0, 2, 4, and 8 weeks at 25ºC was measured and is set forth in Table 6 below.

Table 6: Total aggregate after storage at 25°C

Formulation	%Total Aggregate 0 weeks	%Total Aggregate 2 weeks	%Total Aggregate 4 weeks	%Total Aggregate 8 weeks
B	3.9	4.6	4.3	3.4
C	5.4	6.6	6.6	4.8
D	3.4	5.1	6.4	6.1

Aggregation of Formulations at 2-8°C: The amount of aggregate and dimer formed in each anti-VEGF formulation (100 mg/ml) after storage for 0 and 4 weeks at 2-8ºC was measured and is set forth in Table 7 below.

Table 7: Total aggregate and dimer after storage at 2-8°C

Formulation	%Total Aggregate 0 weeks	%Total Aggregate 4 weeks	% Dimer 0 weeks	% Dimer 4 weeks
B	3.9	3.8	3.3	3.2
C	5.4	5.7	4.7	5.1
D	3.4	3.5	2.8	2.9

Aggregation at various Arginine concentrations at 40°C: The amount of total aggregate formed in each anti-VEGF formulation at various arginine acetate concentrations was measured and is set forth in Table 8 below.

Table 8: Total aggregate at various Arginine acetate concentrations

Arginine Acetate Concentration (mM)	%Total Aggregate
25	5.2
50	4.8
100	4.4
200	4.2

[0222] *Effect of Excipients and Ionic Strength:* The effect of different excipients on the stability of anti-VEGF was investigated. A list of excipients explored includes sodium phosphate, arginine acetate, sodium acetate, and histidine chloride. Our results showed that formulations containing arginine chloride and histidine chloride aggregated faster than all other formulations.

[0223] The stability of anti-VEGF was evaluated in various buffer conditions. The data obtained from the study showed that anti-VEGF is more stable in arginine acetate buffers between pH 4.0 and pH 6.0. The data obtained from the formulation screening study showed that anti-VEGF is stable and has reduced aggregate and dimer formation at 100 mg/mL protein concentration in 200 mM Arginine Acetate, 0.04% PS20 at pH 5.2.

CLAIMS

We claim:

1. A stable aqueous pharmaceutical formulation, the formulation comprising a therapeutically effective amount of an antibody in an arginine buffer, pH 4.0 to 6.0.

2. The formulation of claim 1, wherein the buffer is an arginine acetate buffer, pH 4.5 to 5.5.

3. The formulation of claim 1, wherein the buffer is an arginine acetate buffer, pH 4.8 to 5.4.

4. The formulation of claim 1, wherein the buffer is an arginine acetate buffer, pH 5.2.

5. The formulation of claims 2, 3, or 4 wherein the arginine actetate concentration in the buffer is from about 25 mM to about 250 mM.

6. The formulation of claims 2, 3, or 4 wherein the arginine actetate concentration in the buffer is from about 50 mM to about 250 mM.

7. The formulation of claims 2, 3, or 4 wherein the arginine actetate concentration in the buffer is from about 75 mM to about 250 mM.

8. The formulation of claims 2, 3, or 4 wherein the arginine actetate concentration in the buffer is from about 100 mM to about 250 mM.

9. The formulation of claims 2, 3, or 4, wherein the arginine acetate concentration in the buffer is from about 120 mM to about 240 mM.

10. The formulation of claims 2, 3, or 4, wherein arginine acetate concentration in the buffer is from about 150 mM to about 225 mM.

11. The formulation of claims 2, 3, or 4, wherein the arginine acetate concentration in the buffer is about 200 mM.

12. The formulation of claim 1, further comprising a surfactant.

13. The formulation of claim 12, wherein the surfactant is polysorbate.

14. The formulation of claim 13, wherein the polysorbate is polysorbate 20.

15. The formulation of claim 12, wherein the surfactant concentration is from 0.0001% to about 1.0%.

16. The formulation of claim 12, wherein the surfactant concentration is from about 0.01% to about 0.05%.

17. The formulation of claim 12, wherein the surfactant concentration is 0.04%.

18. The formulation of claim 1, wherein the antibody concentration is from about 10 mg/ml to about 250 mg/ml.

19. The formulation of claim 1, wherein the antibody concentration is from about 25 mg/ml to 200 mg/ml.

20. The formulation of claim 1, wherein the antibody concentration is from about 50 mg/ml to about 150 mg/ml.

21. The formulation of claim 1, wherein the antibody concentration is from about 75 mg/ml to about 125 mg/ml.

22. The formulation of claim 1, wherein the antibody is not subject to prior lyophilization.

23. The formulation of claim 1 wherein the antibody binds VEGF.

24. The formulation of claim 1, wherein the antibody is a monoclonal antibody.

25. The formulation of claim 24 wherein the monoclonal antibody is a full length antibody.

26. The formulation of claim 24 wherein the monoclonal antibody is an IgG1 antibody.

27. The formulation of claim 24 wherein the monoclonal antibody is a humanized antibody.

28. The formulation of claim 24 wherein the monoclonal antibody is an antibody fragment comprising an antigen-binding region.

29. The formulation of claim 28 wherein the antibody fragment is a Fab or F(ab')2 fragment.

30. The formulation of claim 24 wherein the monoclonal antibody binds VEGF.

31. The formulation of claim 30 wherein the antibody is bevacizumab.

32. The formulation of claim 1 wherein the monoclonal antibody is susceptible to aggregation.

33. The formulation of claim 2 wherein the buffer is 200 mM arginine acetate pH 5.2, the surfactant is polysorbate in an amount of about 0.01-0.1% v/v, wherein the formulation is stable at a temperature of about 40°C for at least 28 days

34. An article of manufacture comprising a container holding a stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody, an arginine acetate buffer from about pH 4.5 to about 6.0, and a surfactant.

35. The article of manufacture of claim 34, wherein the antibody binds VEGF.

36. The article of manufacture of claim 35, wherein the antibody is bevacizumab.

37. A method for stabilizing an antibody in an aqueous pharmaceutical formulation by combining a therapeutically effective amount of an antibody, an arginine acetate buffer from about pH 4.5 to about 6.0, and a surfactant.

38. The method of claim 37, wherein the antibody binds VEGF.

39. The method of claim 38, wherein the antibody is bevacizumab.

40. A stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody, 200 mM arginine acetate buffer at pH 5.2, and a surfactant.

41. The formulation of claim 40 wherein the antibody binds VEGF.

42. The formulation of claim 41 wherein the antibody is bevacizumab

43. An article of manufacture comprising a container holding the formulation of any one of claims 40-42.

44. The formulation of claim 1 which is sterile.

45. The formulation of claim 1 which is stable upon storage at about 40°C for at least 28 days.

46. The formulation of claim 1 which is aqueous and is administered to a subject.

47. The formulation of claim 46 wherein the formulation is for intravenous (IV), subcutaneous (SQ) or intramuscular (IM) administration.

48. The formulation of claim 46, which is for IV administration and the antibody concentration is from about 10 mg/ml to about 250 mg/ml.

49. The formulation of claim 46, which is for IV administration and the antibody concentration is from about 50 mg/ml to about 100 mg/ml.

50. The formulation of claim 46, which is for SQ administration and the antibody concentration is from about 25 mg/ml to about 250 mg/ml.

51. The formulation of claim 46, which is for SQ administration and the antibody concentration is from about 50 mg/ml to about 100 mg/ml.

52. A vial with a stopper pierceable by a syringe comprising the formulation of claim 1 inside the vial.

53. The vial of claim 52 which is stored at about 2-8°C.

54. The vial of claim 52 which is a 3cc, 20 cc or 50 cc vial.

55. A stainless steel tank comprising the formulation of claim 1 inside the tank.

56. The tank of claim 55 wherein the formulation is frozen.

57. A method of treating a disease or disorder in a subject comprising administering the formulation of claim 1 to a subject in an amount effective to treat the disease or disorder.

58. The method of claim 57 wherein the antibody binds VEGF.

59. The method of claim 58 wherein the antibody is bevacizumab

60. A pharmaceutical formulation comprising: (a) a full length IgG1 antibody susceptible to deamidation or aggregation in an amount from about 10 mg/mL to about 250 mg/mL; (b) arginine acetate buffer, pH 4.5 to 6.0; and (c) polysorbate 20 in an amount from about 0.01% to about 0.1%.

61. The formulation of claim 60 wherein the antibody binds VEGF.

62. The formulation of claim 61 wherein the antibody is bevacizumab

63. A method for reducing aggregation of a therapeutic monoclonal antibody, comprising formulating the antibody in an arginine acetate buffer, pH 4.5 to 6.0.

64. The method of claim 63 wherein the antibody binds VEGF.

65. The method of claim 64 wherein the antibody is bevacizumab.

66. A pharmaceutical formulation comprising an antibody that binds to VEGF in an arginine acetate buffer at a pH from about 4.5 to about 6.0, and a surfactant.

67. The formulation of claim 66 wherein the antibody is bevacizumab.

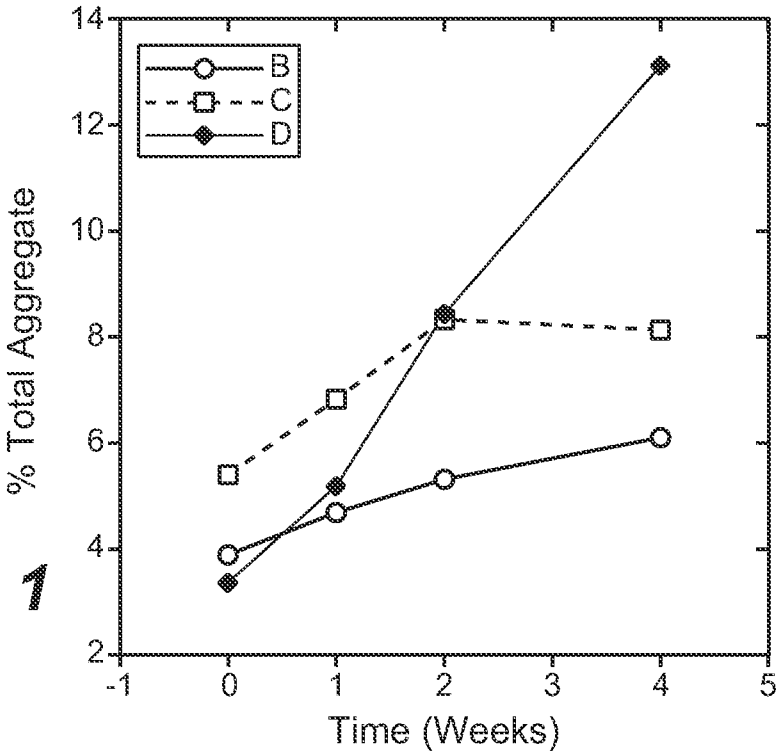
68. A method of making a pharmaceutical formulation comprising:

(a) preparing the formulation of claim 1; and

(b) evaluating physical stability, chemical stability, or biological activity of the antibody in the formulation.

69. The method of claim 68, wherein the antibody binds VEGF.

70. The method of claim 69, wherein the antibody is bevacizumab


B
C
D
% Total Aggregate
Time (Weeks)
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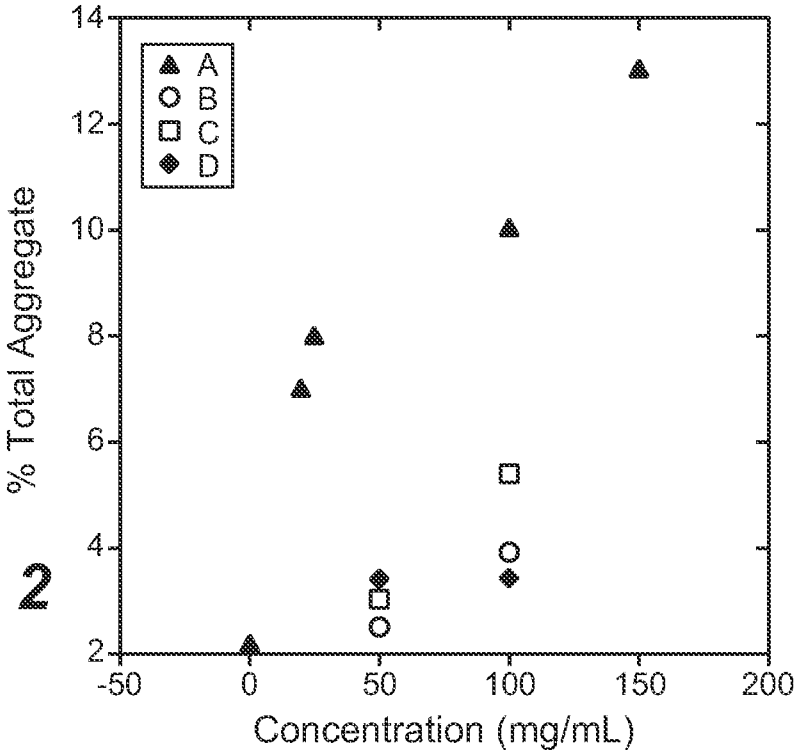
12
10
8
6
4
2
-1
0
1
2
3
4
5

FIG. 1


A
B
C
D
% Total Aggregate
Concentration (mg/mL)
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12
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8
6
4
2
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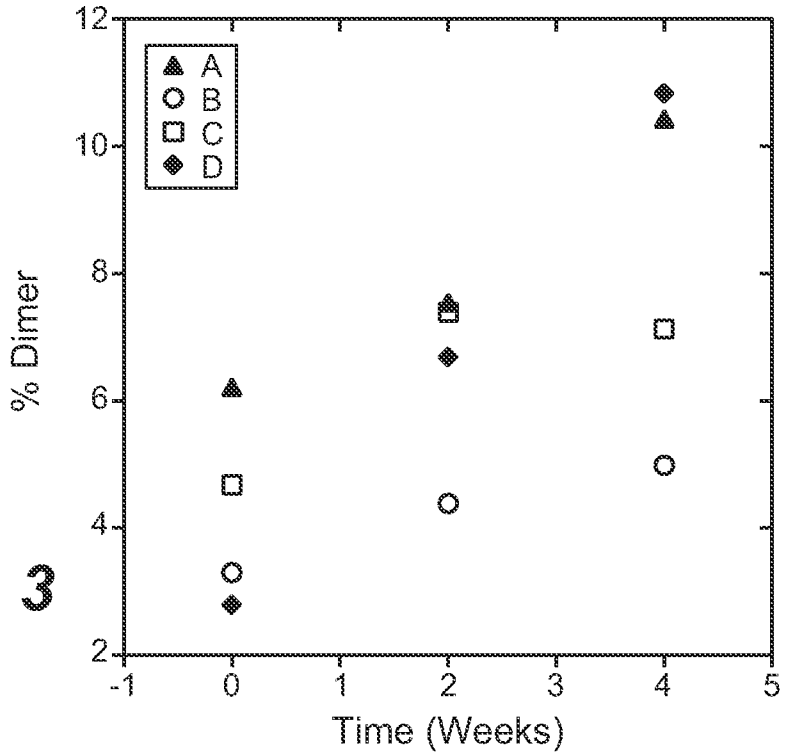
0
50
100
150
200

FIG. 2


A
B
C
D
% Dimer
Time (Weeks)
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10
8
6
4
2
-1
0
1
2
3
4
5

FIG. 3

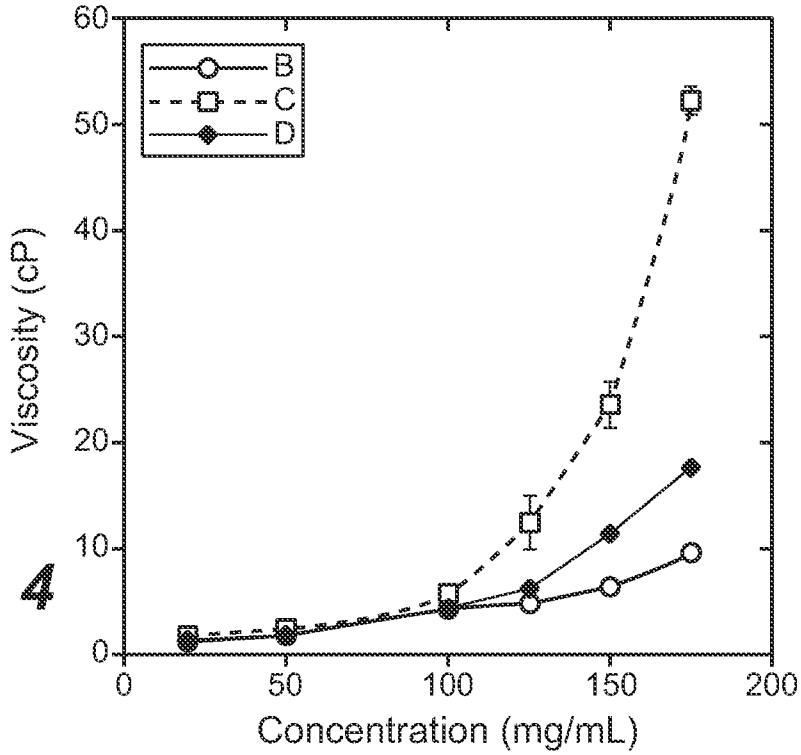
B
C
D
Viscosity (cP)
Concentration (mg/mL)
60
50
40
30
20
10
0
0
50
100
150
200

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/061347

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 A61K47/18 A61K47/26 C07K16/22 A61K9/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/009406 A1 (SMITHKLINE BEECHAM CORP [US]; BRISBANE CHARLENE E [US]; KETKAR AMOL SH) 15 January 2009 (2009-01-15) examples; page 2, line 29 - line 32 page 19, line 27 - page 20, line 10 page 21, line 1 - page 22, line 11; table 1 -----	1-70
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 March 2011	28/03/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Sproll, Susanne

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/061347

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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